HENRY FORD HOSPITAL

International Symposium

# The Leukemias

# Etiology, Pathophysiology and Treatment

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1 The Dynamics of Virus and Rickettsial Infection

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International Symposium

# THE LEUKEMIAS
# Etiology, Pathophysiology, and Treatment

Edited by

**John W Rebuck**
Division of Hematology
Department of Laboratories
Henry Ford Hospital
Detroit

**Frank H Bethell**
The Thomas Henry Simpson Memorial Institute
for Medical Research
Ann Arbor

**Raymond W Monto**
Division of Hematology
Department of Medicine
Henry Ford Hospital
Detroit



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## Participants

| | |
|---|---|
| Awapara Jorge | Houston Texas |
| Berman Lawrence | Detroit Michigan |
| Bessis Marcel C | Paris France |
| Bethell Frank H | Ann Arbor Michigan |
| Bierman Howard R | Duarte California |
| Brown George B | New York New York |
| Buchanan John M | Cambridge Massachusetts |
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| Winzler Richard J | Chicago Illinois |
| Witts L J | Oxford England |
| Zuelzer Wolf W | Detroit Michigan |

## PREFACE

In the last two decades most of the medical disciplines have made dramatic and far reaching advances toward the control and cure of a major share of the diseases which appropriately pertain to them In that portion of medical science which concerns itself with the leukemias the advances have been complex It could hardly have been otherwise Hematologists in the previous generation had achieved a higher insight into free celled leukocytic structure than was true in the tissue organ areas of other disciplines Consequently cytologic objectives whose functional significance was barely appreciated elsewhere were common place here

This volume accomplishes two purposes It gives the present complex position of leukemic etiology and pathophysiologic disturbance a much needed knowledge of detailed leukocytic functions and comprehensive thought on the therapy of the leukemias It establishes a basis of com parison with the earlier works of Downey and Forkner in this field in the late thirties and thus makes it possible to see with some clarity the extent of progress made Actually the progress has been extensive and at an accelerated rate of speed

Perusal of the material contained in this volume suggests some fas cinating and challenging problems ahead When hematology struggled for tools and methods other than the blood smear or the counting chamber its choices and decisions were limited Once this point was passed however each investigator was faced with a new question "What kind of approach do I wish to take?" The hematologist may literally choose for example between electron microscopic immunologic biochemical metabolic microbiologic endocrine genetic toxicologic and isotopic routes Each investigator makes these choices according to his training and objectives This volume details in quiet pages the aggregate achievements stemming from these personal decisions

Coming a century after Virchow s and Bennett s recognition of leu kemia this book as a whole—as well as almost every individual chapter—is in a very real sense the result of cooperative effort The content of this volume arises from the proceedings of an International Sym posium on *The Leukemias Etiology and Pathophysiology* held at Henry Ford Hospital Detroit March 8–10 1956 It is significant that this symposium was suggested and sponsored by the members of the

clinical staff of Henry Ford Hospital and its Executive Director Dr Robin C Buerki It is a pleasure to acknowledge their consideration as well as the initiative and effort of the members of the program committee the fifty six participants and the more than four hundred fellow scientists who were in attendance

*Detroit Michigan*
*January 1957*

J W Rebuck

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## Part VIII Nucleic Acids as the Target for Chemotherapy Mechanisms of Drug Action and Resistance

Chairman Joseph H Burchenal

## Part 1

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Chairman
Wolf W Zuelzer
The Children s Hospital
Detroit Michigan

The Symposium was sponsored by the Henry Ford Hospital Detroit Michigan and held at the Hospital March 8 9 10 1956

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# I

# Electron Microscopy of Normal and Leukemic Leukocytes

MARCEL C BESSIS
National Blood Transfusion Center Paris France

## General Considerations

The ability to make very thin preparations is one of the principal difficulties which interferes with the use of the electron microscope This problem has been solved only since 1954 when a technique for obtaining extremely thin sections was developed

Formerly one had to use the technique of spreading and the technique of casting and shadowing which can be applied to isolated cells only In this way one can obtain excellent pictures of the cell surface This process has led for example to the study of the surface of cells sampled by sternal puncture But the casting technique does not show anything about what is inside the cell, and here lies the enormous advance accomplished by the ultrathin section technique It is now possible to obtain preparations the thickness of which is in the neighborhood of 0 02 μ As a result a white cell may be sliced into 800 sections To get this surprising result took only a few improvements in the usual sectioning technique The first and most important is the use of very hard embedding substances After various researches in the paraffin field embedding in plastic has been adopted the specific substance being butyl methacrylate After dehydration the fragments which are to be sectioned *are soaked in monomeric* methacrylate which is later *polymerized* The necessity of obtaining ultrathin sections led also to special adjustments of the microtomes Ordinary microtomes with mechanical advance were used at first These were replaced by the principle of thermic advances in which the block holder is tied to a metallic rod which is slowly heated by an appropriate apparatus and which expands out in proportion to the heat In this way one avoids the inconveniences of mechanical advance always allowing a certain amount of slackening

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Nevertheless for the time being good results may be obtained with either of these two types of microtomes

* * * * *

It is important to appreciate that electron microscope sections cannot be interpreted in the same way as sections seen with an ordinary microscope A fundamental point which distinguishes them lies in the staining process applied to the latter and this in reality consists of two processes The first is well enough known The proteins which make up the various structures of cells and tissues have variable properties as regards their chemical combination with or adsorption of more or less specific stains The second which is generally overlooked is very important and depends on the fact that Canada balsam (or a similar mounting medium) is used this because of its refractive index suppresses everything which has not been fixed by the stain thus rendering the stained material all the more clear The fundamental technique of classical hematology indeed is to show up structures which stain and suppress those which do not

The structures which are suppressed in this way represent the confused mass of cellular and tissue proteins which have been coagulated and deformed by fixation and which if seen would appear in a very different form from that in which they normally exist These are what are called artifacts One forgets them because one does not see them the observer takes into account only the material which is stained and not the surrounding unstained material which is rendered invisible by the mounting medium Although the unstained material is not seen it nevertheless exists

When phase contrast is used otherwise unperceived differences of refractive index are revealed and these artifacts appear distinctly The importance of structures which classical staining techniques render invisible now becomes apparent and it will be clear that the images furnished by staining techniques are simple and definite but also incomplete

If this is true when ordinary histological preparations are examined with phase contrast instead of the ordinary microscope it is even more true when the electron microscope is used for the latter shows up the smallest artifact due to technique and the important problem of histological fixation has to be looked into anew from the standpoint of electron microscope technique

There is a second fact which must be appreciated and which has to do with the physical conditions under which the image is formed in the

electron microscope With the ordinary microscope the definition of structures is clear only in the focal plane Objects situated above and below this plane are not seen By varying the focus one can change the focal plane and in this way see a variety of different structures This allows one to use sections of a reasonable thickness always with the condition that they are sufficiently transparent

With the electron microscope the conditions are altogether different for the structures in different planes of the section are superimposed as seen either on the fluorescent screen or on the photographic plate This makes it necessary to obtain ultrathin sections without these the electron microscope gives nothing but fuzzy images

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There is a possibility that some day new techniques may be devised which will provide for the electron microscope the same advantages as are produced by staining and mounting in balsam The salts of heavy metals could perhaps take the place of stains by becoming fixed to one or another histological structure and rendering it more opaque to electrons This would be a sort of "electronic staining By treating the fragment of tissue with a substance such as an enzyme or a simple solvent which would dissolve the modified proteins which fog the "electronically stained" structures the latter might be made to present a clear and distinct electron microscope image Such selective dissolving out of material would have the same effect as the use of Canada balsam in classical histological technique

### Considerations Regarding Fixation with Reference to Electron Microscopy

Fixatives or fixing fluids which are considered as good from the standpoint of the ordinary microscope are not generally good from the standpoint of the electron microscope the greater resolving power of which shows up more subtle alterations Further the staining methods used in ordinary microscopy allow the use of fixatives which produce gross coagulation staining methods which reveal only unaltered structures do not call attention to these It should be noticed that the phase contrast microscope which permits observation of the contents of the cell without the use of stains is no better than the electron microscope in concealing the effects of fixation

Indeed as we proceed toward higher and higher magnifications with the electron microscope and toward the perception of smaller and smaller differences in refractive index with phase contrast microscopy

Nevertheless for the time being good results may be obtained with either of these two types of microtomes

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then still be necessary to determine the correct conditions of drying and freezing before comparisons with living cells could be made

Ideal fixation which immobilizes each molecule *in situ* is very difficult to achieve and occurs in special cases only The use of the usual fixatives should accordingly be continued Above all one should require of a fixative that it does not change the spatial arrangements of the soluble proteins and that it does not obscure them by the coagulation products of other soluble proteins Fixatives which produce serious artifacts need not be absolutely rejected provided that they leave some structures for example the mitochondria unaltered and if the constituents which they alter can be eliminated in some secondary fashion for example by digestion Used in this way the methods have a certain resemblance to the classical histological techniques which leave as unstained and invisible all structures which are not shown up by the particular method used Finally the study of the artifacts themselves is often instructive

## Material and Technique

We refer the reader to the works mentioned in the reference list in which the technique is revealed in detail (4 5 29 31 39 47) For the section method the material (blood bone marrow lymph node puncture etc ) is fixed with the solution of Palade (34 35) or of Dalton (23) treated with methacrylate according to the technique of Borysko and Swerdlow (19) and sectioned with the Sjostrand microtome (48) or the microtome of Porter and Blum (46)

The spreading technique and the method for spreading and casting have been described in earlier papers (7 8 12 13) and explained in a recent book (10)

## Structures in Leukocytes Revealed by the Electron Microscope

### The Nucleus

The nucleus does not present appreciable structure on spreading or casting In sections it shows quite unexpectedly an appearance which is not very different from the one observed with phase contrast It is darker than the other parts of the cell and its density depends on the condensation of the chromatin hence young cells have a rather light nucleus whereas in older cells the nucleus appears to be darker It is limited by a dense membrane the thickness of which varies with the different cell species but which is always seen in young cells This membrane sometimes presents some small thickenings which in older cells become more and more noticeable even forming clumps of

the problem of artifacts increases It is no longer sufficient to retain a general outline of tissue and cellular structure it is now necessary to preserve structural detail on a macromolecular scale This is certainly a particularly difficult problem which has been the subject of several *recent investigations*

### The Concept of Fixation as Dependent on the Size of the Object under Consideration

When one considers structures in cells and in tissues which are near the limit of visibility provided by the ordinary microscope (ie as small as 0 2 to 0 1 $\mu$) fixation should mean the coagulation of the sub stances which make up these structures without changing their form—ie they are kept in their place so that there is no displacement visible at the magnification which the ordinary microscope provides Histo logical fixation properly speaking seeks to coagulate the substances of living matter but the coagulation is not accompanied by a displacement of either the original or the coagulated material

The idea of fixation changes somewhat when we enter the region of submicroscopic dimensions from 1000 A to 50 A which is that of molecular structures and investigated with the electron microscope Now one should require that there shall be no displacement of micro molecules and that the bonds which unite them in the living cell shall be preserved Considering only the proteins one is faced with the necessity of avoiding first the separation of macromolecules from each other by breaking down their bonds and introducing water (the arti facts of swelling and of solution) and second the production of masses by creating new bonds (the artifacts of coagulation and syneresis)

Finally a third region of size can be imagined this being the molecu lar region below about 50 A In this region fixation would consist in immobilizing the molecules without changing them so that they could eventually be restored to their initial state for example when the activity of the enzyme molecule is restored to its original state No de naturation of protein molecules would be involved This result is ob tained by only two methods dehydration and freezing *The test which allows one to say whether a cell or a tissue is fixed well or badly is dif ficult to define*

In the case of the living cell the fundamental criteria are clear Un fortunately they cannot be applied at present to observations with the electron microscope One might think that one could use as a reference a cell which was dried in some standard way the constituents being immobilized by drying either preceded by freezing or not It would

small granules their average diameter being about 100 A Others are made up of a network presenting smooth surfaces This last structure had been observed before with the optical microscope particularly by Gonzales Guzman (28) Estable and Sotelo (27) and Zajdela and Morin (53) Electron microscopy has helped to confirm this observation as shown by Borysko and Bang (18) and Bernhard *et al* (2) Sometimes nucleoli show vacuoles especially in pathological cells

THE MITOCHONDRIA

Mitochondria can be found in any kind of cell Up to now hematologists have not attached much importance to them probably because they do not appear on Giemsa stained films Electron microscope studies have shown that in animals and in plants the mitochondria have the same structure and that this structure is rather complex

*In leukocytes mitochondria are sometimes rounded or oval shaped* often elongated or even filamentous Their average thickness is about 0 5 μ Sections made for electron microscopy usually being about 0 03 μ one can understand that the picture which is obtained a bidimensional one does not permit the direct observation of the real forms that the mitochondria occupy in space In order to examine them it is neces

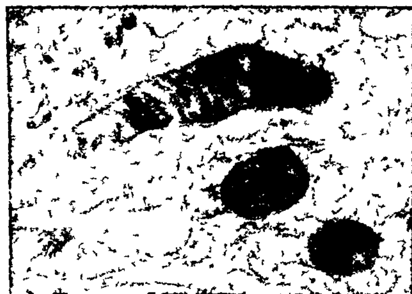


FIG 2 Longitudinal section of a mitochondrion in a plasma cell Note the presence of two dark granules in the matrix These occur frequently

chromatin big enough to be observed with the optical microscope

The inner part of the nucleus is made up of a mass usually not homogeneous in which it is rather difficult to distinguish an orderly structure The nuclear membrane which is described above and which is the only one to deserve such a name is lined with an extremely thin external membrane The latter may be seen only in very thin and greatly enlarged sections

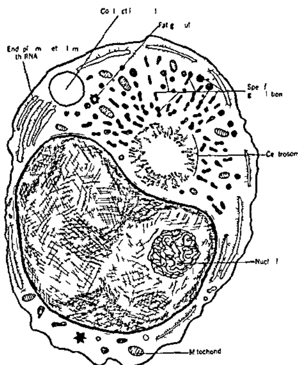



FIG 1 Diagram of a section of neutrophil premyelocyte as seen when observed with the electron microscope

Recently the external membrane and the space limited by the two membranes have been studied extensively This space is now considered as dependent on specific structures which have been revealed by the electron microscope and called the endoplasmic reticulum After describing the endoplasmic reticulum we shall further describe this space

The nucleolus is always darker than the background of the nucleus Its structure varies with different cells depending on their age and perhaps on their functional stage Some of them seem to be made up of



Fig 4 Neutrophilic granulations (casting)

Fig 5 Neutrophilic granulations (section)

sary to reconstitute their structure in space by using sections at several incidences or by examining a series of sections Such reconstitutions have provided the following picture of these small structures (34 49)

Each mitochondrion is limited by a membrane the thickness of which varies between 10 and 25 mμ Toward the inner part of the granule this membrane develops into a structure to which the name cristae or "crests has been given The appearance of these crests varies consider

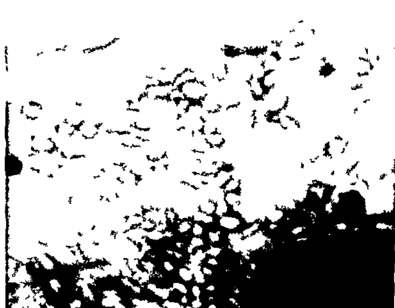


FIG 3 Neutrophilic granulations (after spreading of the cell)

ably with the incidence of section and also it seems with the general shape of the mitochondrion In certain cases the structure appears lamellar in others villous Sometime these lamellae appear quite regularly perpendicular to the mitochondrial surface at other times they do not seem to be oriented Their thickness is about 20 mμ which is about the same as the thickness of the mitochondrial membrane

Greater enlargement shows clearly that these crests and membrane are really made up of two coats each with a thickness of about 8 mμ and in between them there is a clear space of about 5 mμ Here we reach macromolecular dimensions One may consider that each coat which

### Leukocytic Granules

Granules in leukocytes have different forms depending on their nature and their maturity. Neutrophilic granules are elongated and rice shaped as the existing technique has already demonstrated; the sectioning method shows them cut at various incidences which gives a great variety of forms.



Fig. 7. Eosinophilic granulations of the rat. Note the elongated aspect of the inner crystal.

Basophilic granules are usually homogeneous, very dark and contained in a vacuole. In dog mastocytoma certain metachromatic granules present an inner lamellar structure (17) but these are mastocytes and not basophilic granulocytes where for the time being such structures have not yet been described.

Eosinophilic granules present, when they are mature, an internal crystal the aspect of which seems to vary with species.

Immature and nonspecific granules (azurophilic with Giemsa) are spherical and easy to single out from mature granules or any leukocytic granules.

limits the crests or the membrane must be composed of two or three layers of protein or lipoprotein molecules

Membrane and crest limit a space usually denser than the cytoplasm and Palade has called this the mitochondrial *matrix* With the enlargements we have considered here (about 50 000 diameters) it appears

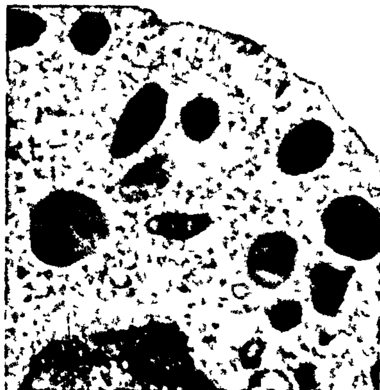


Fig 6 Human eosinophilic granulations

structureless but very dense granules of 2 or 3 mμ in diameter may sometimes be observed

In these granules everything—e g the aspect the dynamism the chemical composition and the universal distribution in every living being—indicates that they must assume a fundamental part in the mechanism of life No doubt these purely morphological researches when they are completed by a study of the functional and pathological states of those small structures will soon lead to a better understanding of the mechanism of their action

which applies to all the cellular formations described in 1905 by Bouin and Garnier in their studies of pancreatic cells and the cells of the salivary glands

In young cells this reticulum appears as flattened bags often kept close to each other in parallel ranks In mature cells it is rather scarce and appears as oval shaped or smoothed formations The works of Palade and of a few other authors have pointed out the fact that the



Fig 11 Lamellae of endoplasmic reticulum in a plasmocyte

walls of these bags are connected on one side with the cell membrane and on the other side with the external membrane outlining the nucleus In sum it is a vast vacuolar network which may open itself at the exterior of the cell and which communicates at least transiently with the perinuclear space When cells are altered by autolysis or immersed in hypotonic medium the reticulum endoplasmic bags swell (Weiss 52) We shall refer to this again later on but now we have to mention certain granules intimately associated with the bag of the reticulum which are known by the name of granules of Palade (36 37) The diameter of these granules varies between 100 and 200 A They absorb electrons rather strongly A great number of them stick to the external face of the endoplasmic reticulum bags They are never seen in the inner part of those bags but they exist frequently isolated especially in mature cells



Fig 10 Zone of the centrosome in a plasmocyte One can discern rather clearly the canaliculi surrounding the centrosome

They probably correspond to the ribonucleoproteins i e to the cytoplasmic basophilic material



Fig 13 A few bags of the endoplasmic reticulum in a leukemic granulocyte

## The Perinuclear Space

We have seen that recent researches with the help of the electron microscope have demonstrated the existence of a membrane belonging to the nucleus and of an external membrane of cytoplasmic origin Between these two membranes lies the perinuclear space which is connected to the bags of the endoplasmic reticulum This space is not completed around the nucleus for one can see certain parts of the nuclear membrane which are directly related to the cytoplasm (51) On the other hand the works of various authors (37 50 51) have demon



Fig 12 Bags of the endoplasmic reticulum surrounded by granules of Palade

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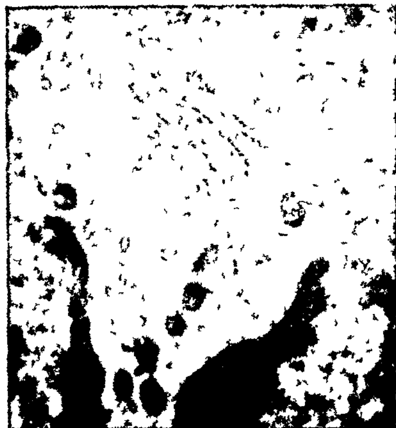


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strated that there is on the surface of the nuclear membrane a series of pores or asperities" at the level of which the two membranes are attached to each other This very complex system is just beginning to be described and is at the moment the subject of active researches

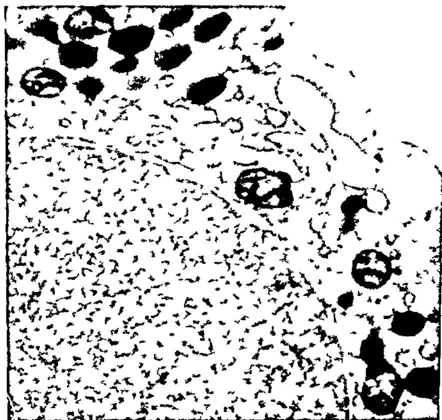


Fig 14 Distention of the bags and the perinuclear space produced by hypotonicity

In so far as blood cells are concerned one may say that this perinuclear space was previously known and had been studied in living cells In many cells after various forms of injury e g the action of certain drugs (26) of autolysis of hypotonicity and of antisera (8 40 41) one can notice the appearance of one or several internucleocytoplasmic vacuoles These vacuoles also called lunules" (26) are juxtanuclear and grow precisely from the perinuclear space Their form is due to the swelling of this space limited by the adhesive properties of these two membranes (Policard and Bessis 43 and in press)



Fig 15 Perinuclear space Top a mitochondrion surrounded by cap of endoplasmic reticulum

Fig 16 Crescent shaped protrusion of the external membrane of the nucleus (Policard and Bessis)

## The Vacuoles

With the electron microscope one can see numerous vacuoles in the cytoplasm of leukocytes the classification of which is still difficult For the time being nothing permits us to distinguish degeneration vacuoles from contractile vacuoles or from the vacuoles of pinocytosis Frequently there are some vacuoles due to the swelling of the endoplasmic reticulum bags these are recognizable by being surrounded by the granules of Palade or because the section shows a continuity between a bag and a vacuole

Here we also might remark on the very special system of more or less dilated canaliculi of the mature megakaryocytes (10) the characteristics of which have not yet been defined and which have not been named

Vacuoles filled with fat appear as very dark spheres This appearance is due without a doubt to the fixation at their level of a great amount of osmic acid Sometimes artifacts due to the coating the sectioning or the fixation cause retraction and give the vacuoles a starlike aspect which is quite characteristic

### Ultracentrifugation of Leukocytes

The examination with the electron microscope of ultracentrifuged cells is very interesting Ultracentrifugation allows the displacement of small structures inside the cell without killing it and thus permits a discrimination between certain artifacts and real structures It is evident that the structures which have been displaced existed in the living cell Furthermore the method allows one to concentrate in a same area of the cell particular molecules or small structures and in this way to emphasize what would have been unnoticed were they more scattered

Let us recall briefly the results of ultracentrifugation of leukocytes examined with the optical microscope (6) Leukocytes have a piriform appearance the swollen part being at the centrifugal pole The nucleus usually occupies a medial position With high centrifugation its effects may be apparent on chromatin the upper pole of the nucleus becomes lighter and the chromatin masses gather at its lower pole The nucleoli can be seen at the lower part of the nucleus The arrangement of granules varies with their nature specific granules settle quite rapidly to the lower part of the cell mitochondria just above and lipid granules are found at the upper part of cell The cytoplasmic basophilia concentrates always in a strictly limited zone above the nucleus when the cell presents granules and below when it does not Sometimes a big vacuole appears at the upper pole of the cell Often colorless with Giemsa sometimes showing a pale pink color this vacuole does not in

dicate either degeneration or cellular death since within an hour the cell resumes an apparently normal activity

Electron microscopy has led to the precise determination of numerous points (9 15)

Small cytoplasmic structures of ultracentrifuged leukocytes settle as follows from the centrifugal pole to the centripetal pole (1) specific granules (2) nonspecific granules (3) mitochondria (4) endoplasmic

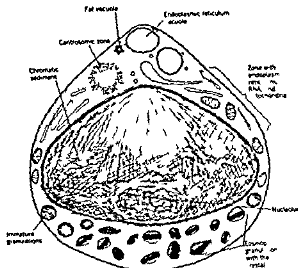



Fig 17 Diagram of a section of an eosinophil promyelocyte after centrifugation

reticulum lamellae (5) vacuoles some of them originating from the swelling of endoplasmic lamellae and (6) fat granules

The nucleus usually settles in the middle of the cell it is often split into two parts the upper one made of nuclear juice the lower one made of dense chromatin These two parts may be entirely separated

The cytoplasmic basophilia usually concentrates above the specific granules In the cells without granules it is at the same level as the chromatin masses Basophilia is not always related to the endoplasmic reticulum

The zone of the centrosome and the Golgi apparatus always settle above the nucleus but below the vacuoles of the apical pole Small vacuoles and canaliculi which form the Golgi body move about as a solid whole

Perhaps the two most important results of these observations are the concentration of basophilia which seems to be due to the granules of

Palade but not always related to the endoplasmic reticulum and the fact that the Golgi body remains unchanged in its morphology and moves about as a whole

## Electron Microscopy of Leukemic Cells

Investigators have attacked the problem of the morphology of the leukemic cell with the electron microscope from two different directions the search for a virus and the search for submicroscopic malformations of the cellular constituents We shall summarize their observations here

### Search for a Virus

As is well known many viruses are visible with the electron microscope not only in the isolated state but also within the cells in certain special circumstances Some investigators have therefore turned their efforts in this direction They have been encouraged by the study on Rous sarcoma cells of Claude *et al* (22) In the hyaloplasm of sarcomatous cells these authors discovered the presence of spherical granules of about 700 A in diameter extremely opaque to electronic radiations and not found in nonsarcomatous control cells Previous ultrafiltration and ultracentrifugation studies (21) had shown that the infective particle must have precisely these dimensions These observations were therefore acclaimed

In 1950 Oberling *et al* (33) described similar granulations in human leukemic cells and put forward the hypothesis that this was the leukemic virus Shortly afterward however it was admitted that comparable appearances were to be found in normal cells (1 14)

We may therefore say that the search for a virus in leukemic cells has for the moment received a setback This does not imply however that the virus is not there for it might be concealed (in the nucleus for example) or it might pass through phases in which it forms part of the host cell proteins and so be unidentifiable As Bernhard *et al* (3) suggest in the case of Rous sarcoma it might only be present in one cell out of 100 or 1000 It might also be represented by one of numerous sorts of granulations of all shapes and size found in the cytoplasm of leukemic cells

There is no a priori reason for thinking that the virus should be any more opaque to electron rays than any other cellular proteins With regard to the Rous sarcoma for instance Claude s studies in particular have shown that the virus has a molecular weight and chemical composition very close to those of the ribonucleoprotein microsomes of normal cellular protoplasm It is difficult to see why one should expect

to be able to distinguish the virus in cancer cells from other cytoplasmic particles of identical composition and molecular weight

SEARCH FOR SPECIFIC MALFORMATIONS

*Myeloblastic and Myelocytic Leukemia* The cells of these forms of leukemia have the property of spontaneous spreading on certain car



Fig 18 Leukemic cell with an Auer body (casting)

riers Their hyaloplasmic veil is very thin and therefore can be examined directly

This method and that of grinding and destruction (7 8 10 12 13) allow the majority of known granulations to be recognized and identified

Among all the granulations to be seen in normal and leukemic myeloblasts one is visible under the ordinary microscope and is probably characteristic of leukemic myeloblasts the Auer bodies They are

paracrystalline rod shaped more or less slender lozenges measuring about 0 5 to 7 μ across The electron microscope shows them very plainly It also shows the existence of microcrystals (7) invisible under

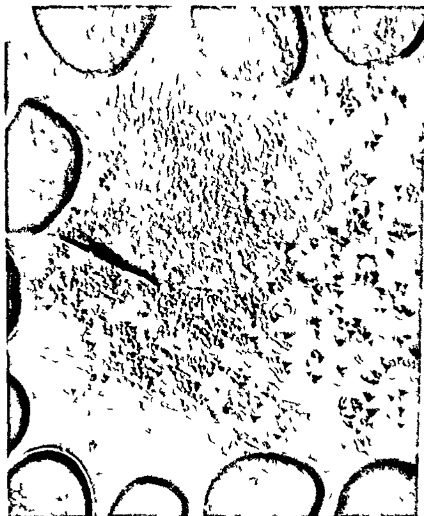


FIG 19 Leukemic cell with an Auer body (after crushing)

the ordinary microscope and these may be found in cells without any large crystals As these formations have so far been observed only in leukemic cells they certainly deserve further study

Details have been obtained about malformations of cells especially the granular anomalies previously described with the ordinary microscope



FIG 20 Rodlets and mitochondria of leukemic lymphocytes

They vary greatly in number from one cell to another some cells have almost none at all whereas others are practically full of them The form of the granulations themselves is very much modified some are two or three times as long as usual Moreover the majority of the cells present very few rod shaped granulations and a large number of round ones

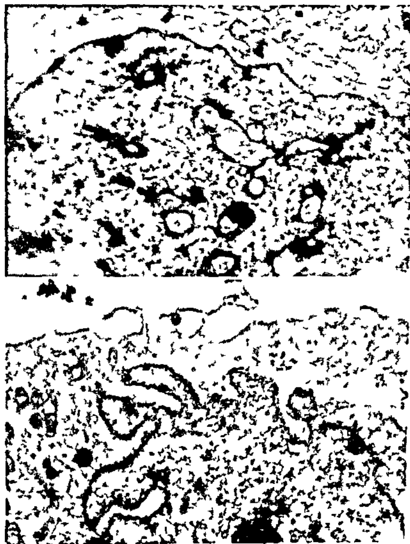


Fig 21 Edge of the nucleus of a Sternberg cell Upper sectioned perpendicularly to the surface Lower tangentially sectioned showing the way in which the cytoplasm seems to indent the nucleus

which is the reverse of normal This probably represents the survival of immature granulations in cells with mature nuclei and is just one of the forms of anarchy in nucleocytoplasmic development

The method of sectioning has given greater precision to the form of these malformations in particular in so far as the nucleoli are con



Fig 22 Nucleoli Beaded appearance containing vacuoles

cerned (cf Figs 22 to 25) Moreover it has shown a few curious formations which we shall provisionally call "crescent like zones" (11)

These are clear spaces the largest of which sometimes measure about 3 by 1 μ Their shape is usually elongated and sometimes bent Their form depends naturally on the incidence of the sections In several cells smaller spaces are found these are smooth and measure about 0.05 μ across The content of these formations presents two principal aspects Sometimes it appears as a rather filamentous mass with a few whirling

points at other times it is made up of granulations of regular dimensions the average diameter of which is around 250 A No dense cytoplasmic structure penetrates them No precise line surrounds them Their limits are therefore well defined by the difference between them and the cytoplasm

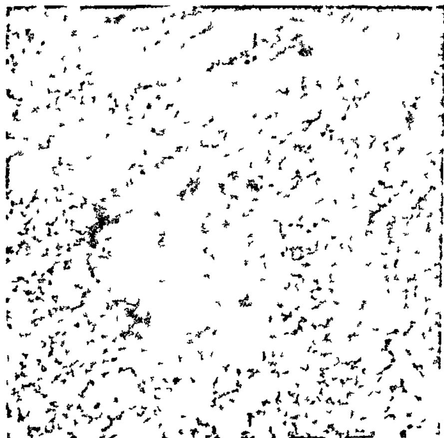


Fig 23 Nucleoli Thinly reticuled appearance

These formations appear in 3 to 30% of leukemic cells They have never been seen in mature cells only in myeloblasts and promyelocytes i e in young stages

These formations are absolutely distinct from the centrosome and the Golgi body One may in the same cell observe the formations side by side

In several cases we have been able to make series of sections which

have shown the very elongated egglike shape of this peculiar zone The hypotony of the surrounding medium which modifies considerably the aspect of the cytoplasm and of the ergastoplasmic formations does not interfere with its appearance

The formations seem to be a form of cytoplasmic degeneration unseen

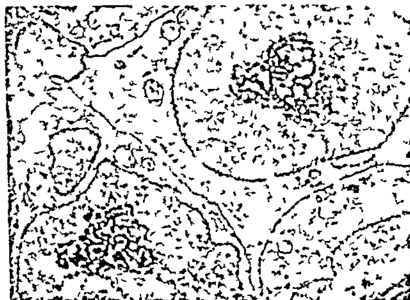


FIG 24 Nucleoli Appearance in loose reticulum

until now in normal cells they do not exist either in all cases of leukemia or in all the cells of the case where they have been observed These zones remind one in many ways of the "degenerative zones" noticed in certain cells affected by a virus such as the zone of degeneration shown by Koprowski (32) Therefore nothing in the present state of our knowledge allows us to advance more than a mere morphological supposition Furthermore there are a quantity of granulations of various sizes not yet classified the importance of which remains to be found

*Lymphoblastic and Lymphocytic Leukemia* The grinding technique has permitted curious formations to be disclosed (7) Among the mitochondria have been observed rods with rounded ends numbering from two to twenty per cell the largest measuring from 1 to 1 5 $\mu$ in length by about 0 2 $\mu$ across and the smallest being considerably beyond the resolving power of the ordinary microscope The rods are sometimes

arranged parallel and very close to each other These formations can not be likened to Auer bodies for they are found in lymphocytes and not in myeloblasts and in chronic leukemia whereas Auer bodies characterize acute leukemia In addition these rods appear to be formed dur

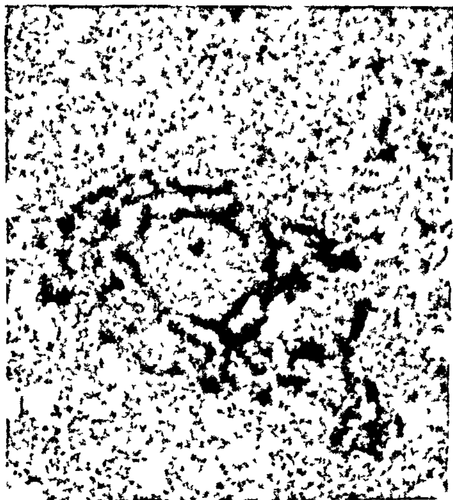


FIG 25 Nucleoli Reticulum appearance with a vacuole

ing the crushing In this aspect only they resemble Charcot s crystals Finally they are found only in some lymphocytic leukemias and never in myeloblastic or monocytic leukemias On one occasion however some were observed in a plasmocytoma

*Mouse Leukemia* In experimental leukemia of AK mice various authors (25a 30) after destruction of the cell have described the presence of numerous particles measuring 100 to 130 A across which they attribute to the possible presence of a virus

The technique of sections (15 16) has shown in addition to the mal



Fig 26 Mitochondria liberated by the destruction of cells these swell and reach enormous dimensions (bottom and right) Compare their size with the ones of mitochondria contained in the cell

formations already described in the nucleus and in the cytoplasm a great number of granules scattered in the cytoplasm the nature of which is unknown (they may be granules of Palade) as well as a few crescentlike zones



Fig 27 Crescent shaped body in a leukemia cell

REFERENCES


1 Bernhard W Braunsteiner H Febvre H L Harel J Klein R and Oberling C *Rev hematol* 5 746 (1950)
2 Bernard W Haguenau F and Oberling C *Experientia* 8 58 (1952)
3 Bernhard W Dontcheff A Oberling C and Vigier P *Bull assoc franç étude cancer* 40 311 (1953)
4 Bernhard W Haguenau F and Leplus R *Rev hematol* 10 267 (1955)
5 Bernhard W and Leplus R *Schweiz med Wochschr* 85 897 (1955)
6 Bessis M *Compt rend soc biol* 144 44 (1950)
7 Bessis M *Acta Unio Intern contra Cancrum* 7 646 (1951)
8 Bessis M "Traite de Cytologie Sanguine Vol 1 Masson et Cie Paris 1954
9 Bessis M *Compt rend* 240 1723 (1955)

10 Bessis M Cytology of Blood and Blood Forming Organs" Vol I Grune and Stratton New York 1956
11 Bessis M and Breton Gorius J Presse med 63 189 (1955)
12 Bessis M and Bricka M Rev hemat l 5 764 (1950)
13 Bessis M and Bricka M Compt rend soc biol 144 654 (1950)
14 Bessis M and Bricka M Rev hématol 6 91 (1951)
15 Bessis M and Thiery J P Rev hemat l 10 583 (1955)
16 Bessis M and Thiery J P Rev hematol 10 606 (1955)
17 Bloom G Friberg U and Larsson B Nord Vet medicin 8 43 (1956)
18 Borysko E and Bang F B Bull Johns Hopkins Hosp 89 468 (1951)
19 Borysko E and Swerdlow M Science 110 66 (1949)
20 Chambers R in E V Cowdry General Cytology University of Chicago Press Chicago 1924
21 Claude A J Exptl Med 84 51 (1946)
22 Claude A Porter K R, and Pickles E G Cancer Research 7 421 (1947)
23 Dalton A J Anat Record 121 281 (1955)
24 Dalton A J and Felix M D Am J Anat 92 277 (1953)
25 Dalton A J Kahler H Striebich M J and Lloyd B J Natl Cancer Inst 11 439 (1950)
25a de Robertis E Canzani R Casic G and Epstein B Blood 10 325 (1955)
26 Dustin P Jr Acta clin belg 4 70 (1950)
27 Estable C and Sotelo J R Inst invest cient biol publ 1 105 (1951)
28 Gonzales Guzman I Le Sang 20 225 (1949)
29 Grey C E and Biesele J J Rev hematol 10 283 (1955)
30 Gross L McCarty S and Cohen I J Cancer Research 12 267 (1952)
31 Kautz J and de Marsh Q B Rev hematol 10 314 (1955)
32 Koprowski H Ann N Y Acad Sci 63 895 (1956)
33 Oberling C Bernhard W Braunsteiner H and Febvre H L Bull Assoc franç etude cancer 37 1 (1952)
34 Palade G E Anat Record 119 427 (1952)
35 Palade G E J Exptl Med 95 285 (1952)
36 Palade G E J Biophys Biochem Cytol 1 59 (1955)
37 Palade G E J Biophys Biochem Cytol 1 567 (1955)
38 Palade G E and Porter K R J Exptl Med 100 641 (1954)
39 Pease D C Rev hematol 10 300 (1955)
40 Policard A and Bessis M Rev hematol 8 57 (1953)
41 Policard A and Bessis M Exptl Cell Research 4 202 (1953)
42 Policard A and Bessis M Exptl Cell Research 8 583 (1955)
43 Policard A and Bessis M Compt rend 242 2496 (1956)
44 Porter K R J Exptl Med 97 727 (1953)
45 Porter K R J Histochem Cytochem 2 346 (1954)
46 Porter K R and Blum J Anat Record 117 685 (1953)
47 Rebuck J W and Woods H Blood 3 175 (1948)
48 Sjostrand F S Experientia 9 114 (1953)
49 Sjostrand F S Exptl Cell Research 9 393 (1954)
50 Watson M L Biochim et Biophys Acta 15 475 (1954)
51 Watson M L J Biophys Biochem Cytol 1 257 (1955)
52 Weiss J M J Exptl Med 98 607 (1953)
53 Zajdela F and Morin C A Rev hematol 7 628 (1952)

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7 Bessis M *Acta Unio Intern contra Cancrum* 7 646 (1951)

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9 Bessis M *Compt rend* 240 1723 (1955)

blasts We were in hopes that this technique would enable us to see a patient with acute leukemia in which we would be unable to identify positively the type of cell that the blast is and then subject it to sectioning and identify the cell We admit that we cannot do this as yet

I felt for a while that the lymphocytes had a more prominent collection of nuclear material at the periphery of the nuclear membrane When the cell is a little bit more mature this is true but in most immature forms of the blast cells the nuclear material looks the same to us in both types of leukemia We are a little disappointed in this but we have been unable to distinguish between blasts with phase for similar reasons

We are interested in plasma cells and we feel that identification with electron microscopy is important In a patient who had multiple myeloma and was doing very poorly the cells were exceedingly immature

In the more mature cells of which you have all probably seen publications the endoplasmic reticulum lies in folds This makes identification easy Most myeloma cells have this appearance too but in most immature cells the amount of endoplasmic reticulum decreases so that the cell is not so easy to recognize as a plasma cell as the more mature forms are

Another patient had a marrow filled with plasma cells and when we sectioned them we could not recognize any and suddenly we realized that actually we were seeing very immature cells

Dr Palade at the Rockefeller Institute is making a review of the findings of endoplasmic reticulum in all types of tissue in which it has been described and it is in every cell as far as we know The material takes on two forms one in which there is a whole series of membranes as you will see in the mature plasma cell and one in which it is separated and forms ovoid or circular bodies This is further divided into the "rough and smooth" form in which there is a smooth outline to the endoplasmic membrane and the "rough" form in which the membrane is composed of a series of small dots called particles of Palade The rough form is designated as ergastoplasm to conform with the older description of this material and endoplasmic reticulum for the smooth form Whether these terms will persist in the nomenclature I do not know

Palade further relates the endoplasmic reticulum to the membranes of the cell In other words it is in close association to the cell membrane and to the nuclear membrane Apparently it plays an important role in the functions of the cell In this respect the folds of the endoplasmic reticulum are more prominent in plasma cells than in any other cell in

# Added Comment

Q B DE MARSH
University of Washington Seattle Washington

We agree essentially with the things that have been described by Dr Bessis in the nucleus of the lymphoblasts the nuclear membrane the changes in the nucleolus and the content in general of the cytoplasm

The mitochondria are of the type Dr Bessis described We think (and I believe most people agree) that in all electron sections of other types of tissue the cristae are positive identification of the mitochondria

Another point in these cells is that we dont see the number of mito chondria in granulocytes nor do we see the amount of endoplasmic reticulum also called ergastoplasm in human cells as has been described in animal cells That is also true of the material we have on megakaryo cytes We do not see the same number of platelet membranes and so on in megakaryocytes in human material as we see in animals

The red cells do not section well with this type of fixation When we started three or four years ago we had a great deal of difficulty in find ing a fixative in which we could fix white cells and get good results We tried formalin fixation which was successful on red cells but it is not very satisfactory for white cells Using Palades suggestion of 1% os mic acid fixative we remove the material from the marrow or peripheral blood and fix it immediately Rapid fixation is absolutely essential if artifacts are to be kept at a minimum We feel after three and a half years of this that most of what we see in these cells are reliable findings and are not artifacts

In myeloblasts of acute myelocytic leukemia we find the same things as described by Dr Bessis but we do not find the area that he described in these cells peculiar to myeloblasts We have noted his publication on this which is a recent one We have gone through all our sections on acute leukemias of both types and have been unable to find similar areas

We are also unable in the number of cases of acute leukemia that we have observed to tell the difference between lymphoblasts and myelo

# 2

# Phase Contrast Microscopy of Leukocytes

SVEN MOESCHLIN
Medical Department Burgerspital Solothurn Switzerland

Phase contrast microscopy has been adopted in the last fifteen years as one of the best methods for examination of living cells and bacteria and has proved even better and more accurate than darkfield illumination Jones (36 37) was the first to report findings with this method in hematology He especially studied the erythroblasts and showed that mitochondria could easily be demonstrated in the cells as typical dark granulations round the nucleus corresponding to the clear zone in the cytoplasm Since his reports about forty papers have appeared concerning the study of the blood cells and the cells of the blood forming organs (1 7 18 20 24 26 34 38-44 45-47 49 53) Albertini (2) was the first to use the method in experimental pathology for the study of tumor cells Zollinger (55 59) has also made extensive studies of normal and pathological cells of different organs and we ourselves have been using the method in hematology since 1944

*Method* A very small drop of blood or of the punctured organ (lymph nodes spleen bone marrow) is put on a clean fine slide (normal glass slides can be used but the best results are obtained with fine quartz slides) This drop is covered with a clean large cover slip and a slight finger pressure is applied for a moment on the surface of it in order to flatten the cells as much as possible The best results are obtained if the layer of cells is very thin and flattened The cover slip is then sealed at the edge with a fine layer of Vaseline to avoid evaporation and movement of the fluid

*Supravital Staining* Kosenow (40) has demonstrated that in phase contrast microscopy of blood cells the fluorochrome *Acridine Orange* because of a marked affinity for the plasma proteins and its capacity to give a good illumination is very suitable for supravital staining The cell

the peripheral blood or the bone marrow We feel they are in some way related to the production of the proteins by this cell

Something that has not been described previously to our knowledge is a storage plasma cell We have seen this cell both in normal human marrow material and in rat marrow material Its cytoplasm presents big endoplasmic reticular sacs filled with material We don t know whether or not it is protein but these areas do stain basophilically

I might add here that the configuration of eosinophils is different in the rat than in the human material It is blocked more and you do not see the type of crystal formation in human material that is so apparent in the eosinophil of the rat

Now I should like to discuss the capillaries of bone marrow There has been an argument about capillary material in the marrow—what the capillaries are like and whether they are closed or open Our sections of capillaries show the open type in vessels of very small caliber

In viewing the capillary wall we find areas of incompleteness We do not think this is an artifact because we see it very frequently in sections that are otherwise technically good As the thickness of the capillary wall increases we do not see these spaces The theory is now that material can pass from the extravascular cell right through the endothelium and into the capillary space This has been suggested in other types of tissue

In closing I should like to say that although we have found many interesting things in bone marrow sections and peripheral blood sections in leukemia we do not feel we have a diagnostic tool yet We have spent most of our time on normal material to become oriented We may not use these observations clinically except perhaps in plasma cell leukemia or multiple myeloma or if we can find something in the future blast cells that may be applicable clinically but certainly we now are able to correlate some of the anatomical observations with the biochemical observations

# 2

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structure and its changes in living condition can be observed in color very nicely

**Observations with Leukocytes**

Our findings with leukocytes (45) have been confirmed by many other investigators (Ludin 43 Bessis and Bricka 12 15 Feissly and Ludin 26 Ackermann 1 and others) The morphological findings in neutrophils eosinophils basophils monocytes lymphocytes and plasma cells have been described in detail by us (45) and by others (12 43 etc) and recently again by Ackermann (1) For the essential findings we refer the reader to the figures and their legends (Figs 1–10) For further details see the previous literature



FIG 1 Neutrophilic leukocytes phase contrast [Wild Heerbrugg (Switzerland)] Notice the typical fine grayish granulation which is dispersed throughout the whole cytoplasm

Instead of presenting morphological details it may be better to give an account of some of the new findings and possibilities which this method has given us for the study of leukocytes and their precursors in the blood forming organs First I should like to say that I have been surprised at how accurate the previous methods of examination of blood cells in the dried and fixed smears have been This is especially true for our staining methods with vital or postmortal stains So the study of fixed and colored cells will still remain an essential method in hematology On the other hand it should be pointed out that phase contrast



Fig 2 Eosinophilic leukocyte (*right*) shows much darker granulation with granules of varying size in comparison to neutrophil (*left*) Typical of eosinophils is a light area appearing in the center of the granules when the focusing adjustment is moved rapidly back and forth

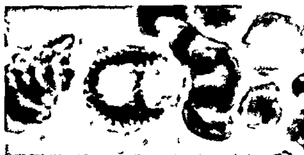


Fig 3 Basophilic leukocyte shows very dark intense granulation the granules being very numerous with almost no space between them The granules are smaller than those of eosinophils and darker than those of neutrophil



Fig 4 Basophilic myelocytes in bone marrow showing the same granulation

microscopy and especially motion picture photography of living cells by phase contrast has enabled us to see more clearly the smaller structures of the nucleus and the cytoplasm

This is particularly so for the mitochondria which could usually not be seen by the common staining techniques and which could only be

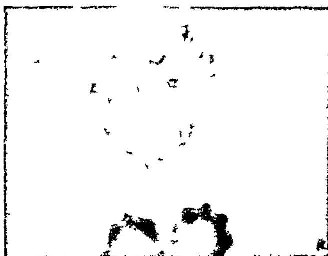


FIG 5 A typical monocyte with kidney shaped nucleus and fine grayish granulation in the cytoplasm The vacuoles probably represent phagocytosed material



FIG 6 Lymphocytes showing fine grayish granulation and less cytoplasm than the monocytes

imperfectly visualized with special stains As Jones (36 37) has already pointed out they usually appear unstained in the colored cytoplasm leaving a clear zone round the nucleus With phase contrast microscopy however these mitochondria can be seen extremely well and the effects of different influences on their behavior can be studied directly under the microscope The centrosome is usually only seen as an ungranulated round spot which shows up very clearly when the cells are filmed with the slow motion camera (see Fig 7) This has been extensively studied by Bessis (11) Franke (27 28) and Ludin (43) The centrosome remains as a moving round spot near the nuclear border

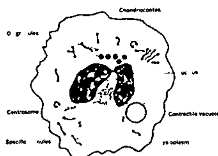



Fig 7 Essential findings in slow film observation of leukocytes For further details see text (From Bessis 11)

and the mitochondria and the granules in the cytoplasm move together from one side to the other with the centrosome with an oscillating movement Also striking are other slow movements of the granules in the neutrophils and the other leukocytes Taken with a slow motion film adapter and then reproduced at a higher speed very interesting movements of the granules can be seen The same is true for the ameboid movement of leukocytes and also for phagocytosis With this film technique very interesting contractive vacuoles studied in detail by Bessis (11) can be demonstrated On a heated slide the details of mitosis of the blood cells can be shown nicely in all its stages

## Special Active States of the Mitochondria

### Lymphocytes

The lymphocytes may show some very bright shining round corpuscles called *Glanzkorner* by Noller (49) in infectious hepatitis At the moment it is not known what they really represent But they are very different from the bright shining agglomerations of round corpuscles

near the nucleus of a megaloblast (*Kugelhaufen*) which we observed (45) and which was confirmed by Czerski and Pawelski (20) Discombe (23) and White (54) (see Fig 9)

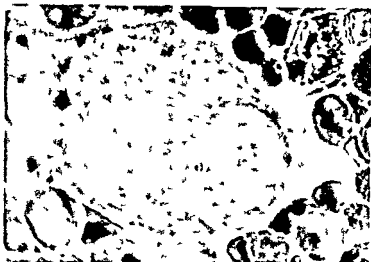


Fig 8 Megakaryocyte—an extremely large cell with fine granulation of the entire cytoplasm The amount of cytoplasm is large when compared with that seen in tumor cells (Hodgkin s etc )



Fig 9 Typical bright shining agglomerations of small granules in the centrosome of megaloblasts With May-Grunwald stain megaloblasts show only a clear unstained area (*left*) in place of the *Kugelhaufen* (Moeschlin) seen with phase contrast (*right*)

RETICULOENDOTHELIAL SYSTEM

*Myeloma Cells* If one studies myeloma cells (45) which are abnormal plasma cells producing pathological globulins mostly of the gamma type one can find enormous dark granules developing round the

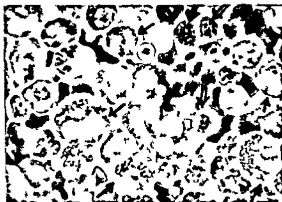


FIG 10 Typical normal bone marrow in phase contrast showing normal neutrophilic myelocytes and metamyelocytes (single arrow) and erythroblasts (double arrow)



FIG 11 Typical myeloma cells showing dark coarse granules around the nucleus probably mitochondria in an especially active phase producing globulin These cannot be seen as granules with normal May-Grunwald stain but sometimes appear as bright areas around the nucleus with the normal stain The same can be seen in plasma cell during immunization

mitochondria in these cells (Fig 11) It is very likely that they are concerned with the production of some form of globulin it may be that they are mitochondria in a special active phase producing these globulins

*Infectious Mononucleosis Cells* These show (45) distinct rather coarse and very irregular dark granules (see Figs 12 and 13) This



Fig 12 Typical mononucleosis Pfeiffer cells of reticular origin These always show a few coarse dark granules of varying size scattered irregularly through the cytoplasm Notice the large amount of cytoplasm in contrast to leukemia cells and the absence of any pronounced nucleoli These dark granules possibly related to globulin production are very similar to those seen in small reticulum cells in bone marrow in active plasma cells and in myeloma cells



Fig 13 Mitosis of the same cell form in the peripheral blood

differentiates them from the lymphocytes (see Fig 6) which never show these large dark granules but have small fine grayish mitochondria We think that these dark granules too may be special active mitochondria producing some globulins The presence of special antibodies in this disease leading sometimes to a positive Paul Bunnell test or to a positive Wassermann reaction is well known This presumption is only theory at the moment but some other findings which we report below make it much more believable It is interesting that these mononucleosis cells may also occur in other diseases—in hepatitis in rubella typically in a South American rickettsiosis and in some drug idiosyn-

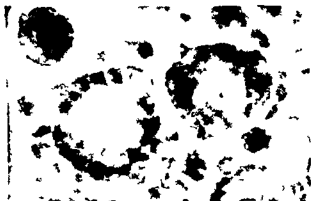


FIG 14 Small reticulum cells from the bone marrow with dark granules around the nucleus probably very active forms of mitochondria perhaps related to some globulin synthesis

crasies such as reactions to phenobarbital and PAS (Cannemeyer *et al* 19) They seem to be a special response to certain reactions connected with the production of some antibodies and not to a specific virus

*Small Reticulum Cells* A special form of large active dark mitochondria very similar to those in myeloma cells can also be seen in reticulum cells of the bone marrow (see Fig 14)

*Plasma Cells* The same granules corresponding to large active mitochondria can be observed in plasma cells and their evolution followed during the process of immunization Since the interesting work of Fagraeus (25) the relation of plasma cells to the production of specific antibodies is well known Our finding of these dark granules probably concerned with this antibody production has been confirmed by Jeschal (33) Hanaoka (30) and Curletto (19a) Jeschal has shown that these granules which cannot usually be seen by the normal staining methods

and come out very clearly with phase contrast microscopy (see Fig 15) can be stained by special methods By methanol fixation one may employ the staining of Regaud Benda or if osmium fixation is used the Altmann Kull method of coloring can be adopted This confirms our hypothesis that these large dark granules are mitochondria in a special active phase producing some form of globulin



Fig 15 Different developmental stages of plasma cells in the red pulp observed under the phase contrast microscope (From Moeschlin *et al* 48 ) (a) Typical transitory form of a reticular cell as it is to be seen principally on the second and third days (b) Mitosis of a young plasma cell Here a few dark granules can already be noted in the cytoplasm (c) Young plasma cell on the fourth day containing numerous dark granules (d) Fully developed half mature plasma cell on the fifth day with numerous dark granules (e) Mature plasma cell on the sixth day Note the decrease of the granules in size and number (f) Old plasma cell on the ninth day without any granules in the cytoplasm

## Experimental Phase Contrast Studies in Plasma Cells during Immunization

In previous work (47) i e in experimental immunization experiments with rabbits we tried to demonstrate that these granules may be related to the production of antibodies Rabbits were first sensitized with an injection of relatively small doses of typhoparatyphoid vaccine Approximately three weeks later when a positive agglutination titer could be demonstrated in the blood an intravenous revaccination with a greater dose was administered This injection leads to a strong plasma cell reaction in the red pulp of the spleen and after several days to a remark

able rise of the agglutinin titer in the blood as was shown by the instructive experiments of Fagraeus (25) Our investigations confirmed the excellent investigations of Fagraeus showing that a remarkable rise of the antibody titer occurs between the fifth and sixth days together with a maximal rise of the plasma cells in the spleen (see Fig 16) Our phase contrast experiments also showed that active immunization causes

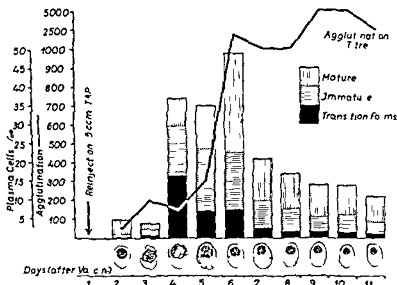



FIG 16 Antibody formation and plasma cell reaction in the rabbit A vast plasma cell reaction appears in the spleen of sensitized animals its peak being on the sixth day (5 × 24 hours) after the reinjection of TAB vaccine The phase contrast microscope reveals an abundance of dark granules in the cytoplasm of the plasma cells the peak being reached on the fifth day These granulations disappear with the appearance of antibodies in the blood and cannot be traced anymore on the ninth to tenth days (From Moeschlin *et al* 48)

analogous changes to take place in the cytoplasm of the plasma cells during the first 5 days just as occur in certain myeloma cells producing pathological globulins (see Fig 11)

At the time of revaccination of the rabbits an impressive plasma cell reaction appears in the red pulp of the spleen before the rise of antibodies in the blood The plasma cell reaction reaches its peak after 5 days The antibodies begin to rise slightly during the first 4 days and the maximal rise occurs after 5 days (that is on the sixth day)

Subsequent to our previous observations on myeloma cells in the phase

contrast microscope we investigated mainly the problem of similar cytoplasmic changes in the experimentally caused plasma cell reaction through the injection of vaccine It was interesting to see that granulations in the cytoplasm quite similar to those in myeloma cells appeared in the reticulum cells (plasmoblasts) during the transformation into young plasma cells The many droplike dark granules in the cytoplasm can be demonstrated by phase microscopy of living unaltered plasma

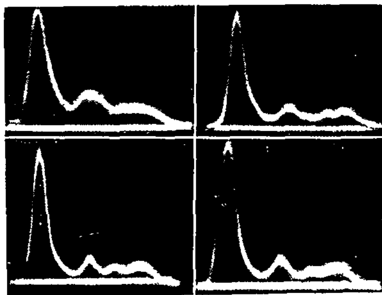


Fig 17 Electrophoretic studies of rabbit serum after typhoparatyphoid immunization on the second fifth and tenth days The gamma globulins show the appearance of a distinct second peak from the fifth day

cells of the splenic pulp These granules appear to be formed by mitochondria which change into these large granules between the second and third days (see Fig 15b) and even more intensively on the fourth and fifth days (see Fig 15c d) These experiments have been confirmed since by Hanaoka (30)

The most striking observation was the fact that these granules quickly reduced in size as well as in number simultaneously with the strong rise of antibodies (see Fig 16) on the sixth day (5 days after the injection of the vaccine) On the eighth to tenth days practically no such granules are to be seen in mature plasma cells and only a few young cells still contain them But this granulation of the cytoplasm of the plasma cells appears mainly in the early phase of immunization It occurs only dur

ing the apparently functional active phase of the plasma cells These changes show a distinct tendency to recede after the appearance of antibodies Electrophoretic studies of the rabbit s serum during typho paratyphoid immunization showed the appearance of a gamma globulin as a distinct second peak after the fifth day (see Fig 17)

In another experiment (Moeschlin and Demiral 46) with phase contrast microscopy we were able to demonstrate that plasma cells taken out of the spleen during the active immunization process may still produce antibodies even if washed several times in saline solution Ob served on a slide under the phase contrast microscope the added typhoid




Fig 18 Washed plasma cells of rabbit spleen on the fifth day after immuniza tion with typhoparatyphoid vaccine These two pictures show two different planes within the same cell (From Moeschlin and Demiral 46 ) (a) Typical plasma cell with many dark large granules probably mitochondria producing antibodies (b) Agglutinated paratyphoid bacilli on the same cell but no agglutinated bacilli on the other cells (lymphocytes etc ) For further details see text

bacilli agglomerated on the surface of the plasma cells and not around the other cells (lymphocytes leukocytes and other reticulum cells) These plasma cells still contained the dark granulation So one may draw the conclusion that these washed plasma cells can still actively produce some antibodies under the cover slide which lead to the ag glutination of the bacilli on the surface of the cells (see Fig 18) Con trols with cell specimens taken out previously or later during the im munization process showed no distinct agglutination

### Tumor Cells

A distinct advantage in the observation of tumor cells by phase contrast microscopy has been demonstrated by several authors (Albertini 2 6 Zollinger 55 59 etc ) The great advantage is that the nucleoli

can be demonstrated sometimes much better than in the fixed and stained preparations This is especially true for the cells of a tumor nature found in gland and tumor punctures in pleural and ascitic effusions and in the blood in leukemia cells More numerous and distinct nucleoli can be demonstrated by this method than by the staining technique (Fig 19) Also the granulations in the cells may appear more obviously and clearly by phase contrast microscopy than by the normal observation in stained films So many blast forms which show no granulations in the

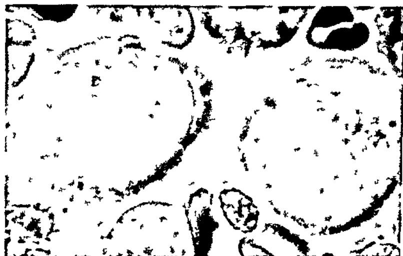


Fig 19 Pathological blast cells of acute lymphatic leukemia showing multiple very large dark gray nucleoli The nuclei show a tendency to lobulation Cytoplasmic granules are sparse coarse and lie mostly about the edge of the nucleus

usual colored slides show distinct granules when observed under phase contrast Auer s bodies which are present rarely in stained smears of myeloid leukemia cells are encountered very often with phase contrast (see references by Ludin Bessis Moeschlin etc ) Brausil (18) in Jugo slavia has drawn attention to the fact that phase contrast microscopy may be helpful for the differential diagnosis of blast forms to see if they belong to the myeloid or lymphatic series As seen in her work the blasts of myeloid origin myeloblasts usually show tiny delicate granules scattered *almost regularly over the whole cell* In contrast however the blast forms from lymphatic and probably also from reticular origin (Moeschlin) show a very scanty granulation lying close to the nucleus This method can be *useful in deciding what therapy steroids* or antifolic drugs should be given We have seen some cases however

where no granulations at all were present and then differentiation may be impossible

*Lymphosarcoma* Phase contrast microscopy has been very helpful in the differentiation of lymphosarcoma from other glandular diseases The lymphosarcoma cells can be clearly differentiated from other small tumor cells as for example bronchus carcinoma cells or Brill Symmers (lymphoblastoma follicular) cells by the presence of very large numerous and distinct nucleoli which show up much more obviously than in the normal stained smears In phase contrast however the cells of one of our gland punctures which were diagnosed by the pathologist in the histological preparation as typical lymphosarcoma cells did not show these typical nucleoli Therefore in this case a diagnosis of small bronchus cell carcinoma was presumed and later confirmed by autopsy In Brill Symmers disease the nuclei are very often lobulated and of different size and show dark zones in the nucleus

#### Summary

The present status of phase contrast microscopy in leukocytes and in their precursors and neoplastic transformations has been reviewed Phase contrast microscopy may be a very useful method for studying the finer submicroscopic structure of the living cells It has also confirmed the accuracy of previous findings with the different vital and fixation coloration techniques and emphasized their continuing value in clinical and experimental hematology Phase contrast filming of cells in slow motion and projection in fast motion have proved to be very useful for the study of special problems in the movement of granules centrosomes mitochondria and leukocytes and macrophages

One of the most essential points and advantages of phase contrast microscopy lies in the very excellent demonstration of the mitochondria This method has enabled the demonstration of special active phases of the mitochondria i e probably as antibody producing structures during immunization

The method is very helpful for the study of tumor cells and enables a better demonstration of the nucleoli and sometimes also of *granules* in these cells This is especially true for leukemia cells in which granules may be seen that cannot be demonstrated by the normal coloring techniques also Auer s bodies may be detected which may not be found by the usual techniques

Phase contrast may be of value in the differentiation between lymphatic blast cells and myeloid blast cells The myeloblast shows a tiny delicate granulation scattered almost regularly over the whole cell

the lymphoblastic leukemia cells more often show a scanty coarse dark granuation lying close to the nucleus

The method may also be applied in the differentiation of lymphosarcoma cells from other tumor cells because of the very numerous large distinct nucleoli seen by phase contrast observation

Thus phase contrast microscopy has proved to be a valuable method in the clinical and experimental study of the blood cells and their precursors and in neoplastic transformations especially in combination with the more standard methods previously employed

REFERENCES

1 Ackermann G A and Bellios N C *Blood* 10 1183 (1955)
2 Albertini A von *Schweiz Z Pathol u Bakteriol* 8 298 (1945)
3 Albertini A von *Schweiz Z Pathol u Bakteriol* 11 701 (1946)
4 Albertini A von *Praxis* 35 No 7 (1946)
5 Albertini A von *Acta Anat* 1 463 (1946)
6 Albertini A von *Rev hématol* 3 139 (1948)
7 Annecke J Medical Dissertation Leipzig 1952
8 Barer R *Mikroskopie* 7 410 (1952)
9 Bessis M *Extr rev hematol* 2 294 (1949)
10 Bessis M *Extr encyclopedie med chir* C60 3 (1952)
11 Bessis M *Blood* 10 272 (1955)
12 Bessis M and Bricka M *Rev hématol* 4 350 (1949)
13 *Bessis M and Bricka M Rev hematol 4 365 (1949)*
14 Bessis M and Bricka M *Rev hematol* 5 764 (1950)
15 Bessis M and Bricka M *Rev hematol* 7 407 (1952)
16 Biedermann F Medical Dissertation Leipzig 1953
17 Bommer W Das Phasenkontrastverfahren in der medizinischen Mikroskopie Kiestner Gottingen 1953
18 Brausil B *Acta Haematol* 12 276 (1954)
19 Cannemeyer W Thompson J R and Lichtenstein M R *Blood* 10 62 (1955)
19a Curletto R *Estr dal Riv Plasma* 1 61 (1953)
20 Czerski I and Pawelski S *Acta Haematol* 13 139 (1955)
21 Dickie A M and Hempelmann L H *J Lab Clin Med* 32 1045 (1947)
22 Discombe G *Rev hématol* 4 481 (1949)
23 Discombe G *Acta Haemtol 3 151 (1950)*
24 Ebersbach W Medical Dissertation Leipzig 1953
25 Fagraeus A *Acta Med Scand Suppl* 9 1 (1948)
26 Feissly R and Ludin H *Rev hematol* 4 481 (1949)
27 Franke H *Folia Haematol* 71 702 (1952)
28 Franke H "Phasenkontrasthaematologie G Thieme Stuttgart 1954
29 Fritze E and Strufe H O *Deut med Wochschr* 35 1076 (1951)
30 Hanaoka M *Acta Haematol Jap* 16 292 (1953)
31 Hansen H G Rominger A and Michel K "Das Phasenkontrastverfahren in der Medizin Vandenhoeck & Ruprecht Gottingen

32 Haselmann H Stoma 4 149 (1951)
33 Jeschal, E Rev "Plasma No 3 (1953)
34 Jeschal E Acta Haematol 10 223 (1953)
35 Jeschal E and Stiller E Z ges inn Med u ihre Grenzgebiete 9 455 (1954)
36 Jones O P Anat Record 100 49 (1948)
37 Jones O P Blood 3 967 (1948)
38 Kabelitz H J Deut arch klin Med 197 369 (1950)
39 Klausewitz, W Natur u Volk 82 283 (1952)
40 Kosenow W Acta Haematol 7 217 (1952)
41 Lawkowicz W Pawelski S and Zawadzki Z A Polski Tygodnik Lekarski 7 33 (1952)
42 Ludin H Schweiz med Wochschr 29 710 (1948)
43 Ludin H Acta Haematol 7 243 (1953)
44 Ludin H Die Organpunktion in der klin Diagnostik S Karger Basel 1955
45 Moeschlin S Acta Haematol 2 399 (1949)
46 Moeschlin S and Demiral B Klin Wochschr 30 827 (1952)
47 Moeschlin S Pelaez J B and Hugentobler F Schweiz med Wochschr 80 1247 (1951)
48 Moeschlin S et al Compt rend 3 congr soc europ hemat I Rome 192 (1951)
49 Noller H G Arztl Forsch 6 185 (1952)
50 Policard A and Bessis M Rev hematol 8 57 (1950)
51 Ralph P H Anat Record 100 73 (1948)
52 Schonenberg H Monatsschr Kinderheilk 99 421 (1951)
53 Schubert E Medical Dissertation Leipzig 1952
54 White J C quoted from Discombe (23)
55 Zollinger H U Mikroskopie 3 1 (1948)
56 Zollinger H U Schweiz Z Pathol u Bakteriol 11 617 (1948)
57 Zollinger H U Schweiz Z Pathol u Bakteriol 11 276 (1948)
58 Zollinger H U Am J Pathol 24 569 (1948)
59 Zollinger H U Am J Pathol 24 545 (1948)

# Added Comment

O P JONES
University of Buffalo Buffalo New York

In reviewing Dr Moeschlin s paper there are certain things that have always seemed to be of the utmost importance with respect to research with phase microscopy as it pertains to leukemic cells I believe Dr Moeschlin was one of the first to use phase microscopy with the idea of describing variations in nucleoli We have known for some time that the nucleoli are important from a metabolic standpoint but their im portance in diagnostic work has been largely overlooked because of the use of May Grunwald Giesma preparations or the dry smear prepara tions in which the presence of nucleoli is questionable They may even be distorted or destroyed They may be concealed and masked by the density of the chromatin

Dr Moeschlin emphasized the importance of studying nucleoli with phase microscopy showing that in lymphosarcoma for example nucleoli are large numerous and fairly dark It is easy to separate these cells from leukemic cells or cells from follicular lymphoblastoma In contrast to the large numerous dark nucleoli in lymphosarcoma he has pointed out that in such conditions as malignant reticulosis there may be two to four dark but small nucleoli In the blastoma cells the nucleoli are small less numerous and lighter These differences are missed in ordinary preparations I feel that his studies with phase microscopy emphasize the fact that we have been overlooking certain of the cellular structures We are now using phase microscopy to interpret the routine prepara tions—to uncover some of the details that have been hidden so to speak

There is another aspect to the utilization of phase microscopy in the study of leukemic cells—the study of the effects of the various technical procedures on the living cells to determine the alterations which are brought about by the fixatives as well as the stains so that by a combina tion of the standard preparations plus phase microscopy we can re evaluate some of the structures we have been looking at for a number of years

In order to emphasize the use of phase microscopy in the study of leukemic cells I have selected material from three or four sources One is from a young lady in Yugoslavia Dr B Brausil (1954) Dr Moeschlin has already presented illustrations from her paper The other material is from articles by Rind and Otto (1954) on the development of a technique for first studying cells by phase microscopy and then studying the same cells with routine procedures either May Grunwald Giesma or Wright fixation

The paper by Brausil is of interest in that contrary to the experience of Dr DeMarsh with the electron microscopy of leukemia cells Brausil with phase microscopy contends that myeloblasts and lymphoblasts can readily be separated from each other If one is unable to establish a diagnosis in routine preparations it can readily be accomplished by phase microscopy of the living cell I am presenting this material primarily because her views are very specific and clear-cut and there may be many who will not agree with them In my own mind I have been wondering whether or not the leukemias she studied were sufficiently undifferentiated or acute so that perhaps there was a difference Another point with reference to her paper is that here are seemingly identical cells in the dry smear preparations stained with May Grunwald Giesma stain which are extremely basophilic and do not have cytoplasmic granules but when Dr Brausil examined the preparations with phase microscopy she noticed quite a difference in the cytoplasmic content of these presumably basophilic or lymphoid cells without granules Dr Moeschlin made this point some years ago—namely cells that appear to be similar in ordinary preparations look quite dissimilar in the living state with phase microscopy Dr Brausil also pointed out that the mitochondria or the granules as she calls them were over the nucleus I want to lead up to some of my work on the influence of the cytoplasmic organoids on the production of nuclear patterns in leukemic cells which are necessary for accurate cellular identification

Illustrations in Dr Brausil's work depict routine preparations of cells which are practically identical With phase contrast Dr Brausil found that in myeloblastic leukemia the granules or mitochondria were finer and more regularly distributed throughout the cytoplasm In the lymphoblastic leukemias they tended to be more rounded plump and larger and they aggregated closer to the nucleus She also pointed out that these cytoplasmic bodies are over and beneath the nucleus but when a dry smear preparation is made from such a cell by flattening it out these bodies are then impressed into the nucleus and contribute to what we have called parachromatin for a number of years Therefore some of

the nuclear pattern, as will be shown later *is contributed by the constituents in the cytoplasm* Nuclear pattern is not a reflection of the nuclear activity alone

Other examples from the acute leukemias which Dr Moeschlin has already shown are paramyeloblastic and paralymphoblastic leukemias The more numerous and finer mitochondria are in the former whereas the larger and more vesicular type are in the latter

Cells from a case that was presented to Dr Brausil for diagnosis are also illustrated The cells were identified originally as myeloblasts in the routine preparation but when studied by phase microscopy they were found to be lymphoblasts So whereas the diagnosis was in doubt in a routine preparation it was made certain by phase contrast microscopy and later it was affirmed at autopsy Dr Brausil has studied over 500 cases of leukemia in 10 years and she attributes her success in diagnosing them to the fact that she flattened the cytoplasm as much as possible before examining the granules (mitochondria) by phase microscopy

*Next I should like to mention that when we study preparations of this type we say* here is a certain cell in a routine preparation Or putting it the other way round here is a cell in a phase contrast preparation what does this cell look like in a routine preparation?

One way of accomplishing this is illustrated in an article published by Rind and Otto in which they describe a technique for the dyeing of phase contrast preparations They examine living cells and then keeping the cell in question within the field raise the objective of the microscope and unseal the preparation as though it were on a hinge with a razor put in fixative and wash and stain it with the cover slip and everything in place After the preparation has been treated thoroughly the cover slip is put down on the slide and the objective lowered The same cell that was being studied with phase microscopy should be stained and in the field

This technique was applied by Weykam (1955) in a study of nuclear configurations of the granulocytes especially the neutrophils With this type of procedure (Phakoprapiratfarbung—which is a contraction for phase-contrast stain preparation) the unstained living neutrophils may be seen with the dark contrast phase microscopy and also an outline of the nuclei Following the same cells through we may see it with phase plus staining as well as with brightfield microscopy after staining

Weykam contended that in routine preparations there is a faithful reproduction of the nuclear configurations as seen in the living cell either by *supravital or phase contrast preparations* The only difference

is that the fixative methyl alcohol causes some cell shrinkage This is one way of solving the problem of studying living cells in one preparation and staining them in a different way in another preparation and then correlating the findings With Rind and Otto s technique the same cell may be studied under different conditions

Some years ago I tried to accomplish this with a little different idea in mind I wanted to find out what happened to dry smears during the staining and fixing processes They were mounted in a drop of 10% formalin in order to apply subsequently other staining techniques Bright and dark phase contrast pictures of an unstained erythroblast show the mitochondria We took the same cells on a cover slip preparation marked them with a diamond point and then treated them with Sudan black or stained them in various ways We even stained them with Wright s stain and then examined them with a brightfield microscope Mitochondria are seen in the Wright s preparation as negative or light images A considerable amount of shrinkage was noted These preparations were originally dry smears

One of the reasons for doing this was to dissect the cells more or less optically and find out what produces nuclear pattern or contributes to it *The main problem was to determine what happens in order to produce* nuclear patterns like those shown in the plate of Dr Downey s on "Leukemic Reticulo Endotheliosis published in his Handbook They have a peculiar cytoplasm and characteristic nuclear pattern By making dry smears mounting them in formalin and studying them with phase contrast it is possible to make optical sections above through and on the other side of the nucleus such as are described below

In dry smears of lymphatic leukemia the nucleus is flattened out with mitochondria or cytoplasmic organoids impressed into it from both surfaces in varying degrees The blood from the same patient was studied in a routine stained preparation Now if we go back to these routine preparations and study the nuclear pattern we can trace things in the nuclear area namely the light areas and find that some of this pattern has been contributed by the cytoplasm

In a similar preparation but from a case of acute myeloblastic leukemia optical sections above through and on the other side of the nucleus are seen by means of camera lucida drawings with dark contrast phase miscroscopy Cells stained with May Grunwald Giesma from the same patient show at one point the cytoplasmic organoids coming in and forming a little bay over the edge of the nuclear membrane There is a shelflike projection of nucleus beneath These organoids were car

ried in over the nucleus and gave rise to some of the pattern which has been called parachromatin

I have been particularly interested in cells from patients with leukemic reticuloendotheliosis Optical sections above through and on the other side of the nucleus from such a case can be obtained The case we studied is like the ones Dr Hal Downey depicted in his Handbook

Optical dissection shows us that most of the patterns or outlines of light material in the cytoplasm can be explained by the presence of underlying organoids Similar images can be found over the nuclear area Therefore the nuclear pattern of cells in these leukemias especially the very acute ones is not a reflection of the activity of the nucleus alone but is a reflection of what is going on in the cytoplasm as well as the nucleus

## General Discussion

Dr George O Gey (Baltimore Maryland) As one who has spent considerable time and effort in using phase techniques and in comparing phase techniques with electron microscopy in examining various cells I should like to make just a few comments regarding the papers that have been given

I think a great effort should be made to simplify our concepts of the dynamics of cell behavior Some of Dr Bessis s schematic representations really leave us in the woods without seeing the trees at least at times There are some simple evaluations that I think can be introduced here and one is that all cells have plasma gel membranes which rapidly incorporate materials from the environment and much of what we see inside cells if we study them with motion pictures and phase microscopy can be shown to be derived from the surface membranes This was mentioned by one of the speakers in connection with the interpretation of the ergastoplasm or of the endoplasmic reticulum as being related to the plasma gel membrane

Many of the various bodies which one finds in these cells are indeed in some cases derived from the environment and often especially in degenerative conditions as a result of phagocytosis

Also our interpretation of the presence of crescents might be considered seriously as evidence of these cells having phagocyted such materials We should not consider all such materials as special organelles which are specific to the cell s organization

The process of using thin sectioning and ultracentrifugation can give us a lot of valuable information regarding differences in organelles One might add here that we can ultracentrifuge living cells for many hours at body temperature without apparently harming them at all even when the gravitational forces are in excess of

100 000 × *g* I am delighted that we have had an opportunity to see how complex this problem can become

DR S DECARVALHO (Cleveland Ohio) Since I did not hear this mentioned I should like to point out a technique for phase microscopy which was developed by Barker in Oxford for the distinction between free nuclei and intact small cells

It consists in suspending the material in 28% bovine albumin Since albumin does not penetrate the cell the cell membrane is clearly visualized The halo is reduced and the amount of cytoplasm relative to the size of the nucleus is much more evident which gives a better idea of the nucleocytoplasmic ratio

In our experience this technique proved very helpful in distinguishing lymphoblasts from myeloblasts and other details in cell structure and in controlling nuclear preparations

# 3

## Antigenicity of Leukocyte Fractions[1]

STUART C FINCH
Yale University School of Medicine New Haven Connecticut

Our initial interest in leukocyte antibody was derived from attempts to demonstrate the mechanism of the L E phenomenon through the use of heteroimmune leukocyte antisera Rabbit sera prepared against human leukemic granulocytes produced *in vitro* agglutination lysis and phagocytosis of normal human granulocytes by monocytes and polymorphonuclear leukocytes Many of the cells produced on incubation with this antileukocytic serum closely resembled L E cells but certain differences in action of human L E factor and leukocyte antibody were noted In any event the use of this experimental antiserum demonstrated clearly three mechanisms by which leukocytes may be altered and rendered susceptible to removal from circulation in the presence of leukocyte antibody

Evidence now is accumulating rapidly that leukocyte antibody is responsible for the production of many human leukopenic states This includes those observed in newborn infants chronic idiopathic agranulocytosis chronic inflammatory disorders and after either the ingestion of certain drugs or the administration of multiple transfusions The immunologic mechanisms involved probably are similar to those involving the erythrocytes (e g acquired hemolytic anemia) and platelets (e g thrombocytopenic purpura)

It is recognized that it would be of considerable value to have available potent cell type specific antisera for the purpose of identifying immature leukocytes and treating certain leukocytic disorders and for the ultimate problems involved in the preservation and transfusion of

[1] This discussion includes observations from studies done in collaboration with Drs Joseph F Ross Franklin G Ebaugh Jr Antonio Cajano and J W Hollingsworth

leukocytes Experimental leukocyte antisera possess considerable cell type specificity but it has not been known which portion of the leuko cyte is responsible for its antigenic properties In order to investigate this problem further studies were undertaken to determine the capacity of leukocyte nuclei and cytoplasms to provoke antibody responses and

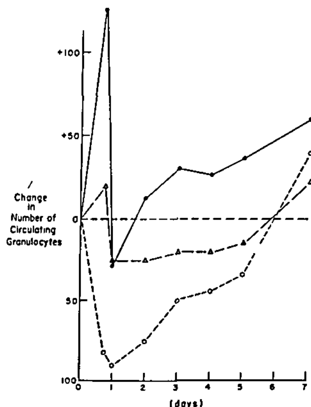



FIG 1 The average per cent change in the number of circulating granulocytes for each group of guinea pigs after the injection of normal or leukocyte fraction antisera Each group contained a minimum of *10 animals* •——• *Normal Rabbit* Serum Δ——Δ Nuclear Antiserum o– – –o Cytoplasmic Antiserum

to evaluate the hematologic effects of each antiserum *in vivo* Antisera to particulate fractions of guinea pig splenic cells and peritoneal exudates were prepared in rabbits Potter homogenization and differential centri fugation methods were employed for the separation of the nuclear and cytoplasmic components Single intraperitoneal injections of either 2 ml or 3 ml portions of each antiserum were administered to 5 normal guinea

pigs and frequent hematologic studies were made during the ensuing 10 days A significant and persistent granulocytopenia was produced in all animals which received the heteroimmune granulocytic cytoplasmic antiserum (Fig 1) The response to the administration of granulocytic

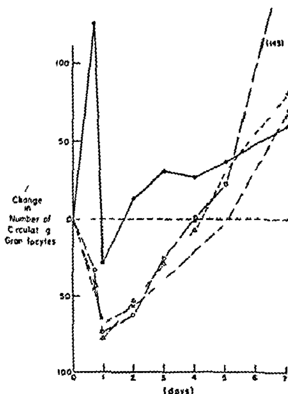



FIG 2 The average per cent change in the number of circulating granulocytes for each group of guinea pigs *after the injection of normal or leukocyte fraction anti* sera Each group contained a minimum of 10 animals •——• Normal Rabbit Serum o——o Mitochondria Antiserum Δ - -Δ Microsome Antiserum x— —x Supernatant Antiserum

nuclear antiserum was much less striking Although little lymphopenia developed immature lymphocytes appeared in the peripheral blood of guinea pigs in response to the administration of splenic cell nuclear antiserum A persistent increase in the number of circulating granulocytes was observed after the injection of splenic cell cytoplasmic anti

serum In contrast no consistent or persistent depression of the number of circulating leukocytes occurred in guinea pigs which had been injected with similar amounts of normal rabbit serum (Fig 1) These studies seemed to indicate that the antigenic properties of guinea pig granulocytes reside principally in the cytoplasmic portion of the cell

Additional studies then were made in an attempt to compare the antigenic activities of subfractions of granulocyte cytoplasm From guinea pig granulocytes the mitochondria microsome and supernatant protein fractions were prepared through differential centrifugation and injected

| TYPE OF SERUM | SALINE DILUTIONS OF INACTIVATED ANTISERA | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1:1 | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1280 | 1:2560 |
| MITOCHONDRIA #1 | + | ++ | +++ | + + | ++ + | ++++ | ++ | ± | 0 | 0 |
| MITOCHONDRIA #2 | | ++++ | ++ | ++++ | ++ | | | ± | 0 | 0 |
| MICROSOME #1 | + + | + + | ++++ | ++++ | +++ | + | ± | 0 | 0 | 0 |
| MICROSOME #2 | | + | +++ | ++++ | +++ | ++ | + | ++ | ± | 0 |
| SUPERNATANT #1 | | ± | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| SUPERNATANT #2 | + + | + ++ | + | + | ± | 0 | 0 | 0 | 0 | 0 |
| NORMAL | + | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Fig 3 Granulocyte agglutinin titers to granulocyte cytoplasmic antisera

into normal rabbits The rabbit antisera produced were tested both for their leukocyte agglutinating capacity and for their ability to induce granulocytopenia *in vivo* From 4 to 6 normal guinea pigs were given intraperitoneal injections of one of the antisera and frequent hematologic observations were made over the course of the next 7 days A profound granulocytopenia developed in practically all the animals given any one of the cytoplasmic fraction antisera In most instances it persisted for periods up to 3 or 4 days and was followed by a polymorphonuclear leukocytosis (Fig 2) For the *in vitro* agglutination studies serial saline dilutions of inactivated aliquots of each antiserum were used Fresh peritoneal exudate leukocytes were used as the antigen in a concentration of about 35 000 per cubic millimeter Except for the low agglutinin titer of the final protein supernatant antisera all the others correlated closely with the *in vivo* response (Fig 3) From this study it was concluded that all three granulocytic cytoplasmic fractions prepared by this method have comparable antigenic properties

The fractionation method employed for these studies certainly did

not produce entirely pure fractions Each of the particle fractions studied probably contained some of the smaller components It is quite clear however that each particle fraction contained little or none of the larger particles For example the mitochondria fraction contained no nuclei but some microsomes With these considerations in mind it is apparent that most of the leukocyte antigenicity is localized in the cytoplasm but probably is not restricted to any one of its individual components

Future progress in the field of leukocyte immunology appears to depend on the development of improved techniques for the detection of leukocyte antigen antibody reactions It is firmly believed that a complete understanding of leukocyte immunologic reactions is the key to understanding the mechanism of many clinical leukopathic states

# 4

## Some Studies on Leukocyte Agglutinins[1]

JOHN J BUTLER
St Mary's Hospital Rochester New York

Agglutinins have been demonstrated in the sera of patients with acquired hemolytic anemia (3) and thrombocytopenic purpura (1 7) and these have been distinguished from naturally occurring anti red cell (8) and antiplatelet agglutinins (12) Leukocyte agglutinins have been demonstrated in various clinical conditions including leukemia (5 6) Some of these diseases are listed below Some authors have implicated these agglutinins as the causative factors for the leukopenia in these diseases

- Neutropenia
- Neutropenia with hemolytic anemia
- Neutropenia with thrombocytopenic purpura
- Chronic pancytopenia
- Pyramidon sensitivity
- Sulfapyridine sensitivity
- Virus pneumonia
- Infectious mononucleosis
- Lupus erythematosus

The purpose of this presentation is to describe some studies concerning the naturally occurring leukocyte agglutinins

Goudsmit and van Loghem (6) tested the sera of 100 healthy donors against type O white cells They found weakly positive reactions in 10 instances Dausset *et al* (5) studied the sera of 300 normal donors for the presence of agglutinins to compatible leukocyte suspensions and failed to demonstrate any

Dausset (4) cites some unpublished experiments in collaboration with van Loghem in which combinations of 50 normal sera and 50 correspond

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ing varieties of white cells were tested for leukocyte agglutination They observed frequent but inconstant agglutinations in the combinations which were incompatible for the A B and O system

Moeschlin and Schmid (10) tested blood samples of 30 persons in 380 leukocyte serum combinations without regard for A B and O blood groups They noted leukocyte agglutination in 60 cases of the 380 combinations which were incompatible with respect to the A B and O blood groups and they concluded that this agglutination was caused by the blood group specific iso agglutinins This conclusion was drawn in *spite of the absence of leukocyte agglutination in two thirds of the cases* in which on the basis of the intensive erythrocyte agglutination they would have expected leukocyte agglutination The authors thought it improbable that a low serum titer of group specific erythrocyte agglutinins *could be responsible for the absence of leukocytic agglutination*

### Methods

Plasma was handled with sterile technique and stored at —20 until used Sequestrene was the anticoagulant throughout All glassware was siliconized and the metal parts were coated with Arquad each time the equipment was used The silicone was stripped from the glassware after each usage by soaking in 10% NaOH solution Blood was collected for the preparation of white cells in the following manner A needle was inserted in the antecubital vein and blood was allowed to flow up to the mark of a 15 ml centrifuge tube containing 1 ml of 1% Sequestrene and 1 25 ml of dextran having an intrinsic viscosity greater than 0 41 The centrifuge tube was covered with Parafilm and inverted gently three times The blood was allowed to settle for about 30 minutes and the supernatant was removed The white cell counts were usually between 10 000 and 15 000 per cubic millimeter This plasma contained platelets and about equal numbers of red cells and white cells The plasma samples and leukocyte suspensions were adjusted to pH 6 9 by the addition of 1/9 *N* citric acid Methyl orange was used as an indicator and about 1 drop of acid was required for each 0 4 ml of plasma and about 0 3 ml of acid for each 7 ml of leukocyte suspension

When a diluent was required in preparing serial dilutions a nonreacting type A plasma of low titer was used after it had been absorbed with type B cells

Plasma leukocyte agglutinin tests were performed by mixing 0 15 ml of white cell suspension with 0 40 ml of test plasma and 1 drop of 30% bovine albumin in a 10 × 75 mm serological test tube This was incubated in a water bath at 37 and read after 2 hours Each test for leuko

cyte agglutination was performed on at least two and in some cases three different occasions The leukocytes were resuspended after incubation by shaking the test tube quickly about six times A drop was poured on a glass slide and allowed to flow out in a thin layer by tilting the slide The slide was studied for white cell clumping under the low power of the microscope either with reduced light or with phase microscopy

Anti A and anti B blood grouping plasmas were prepared as follows About 5 g of a potent dried anti A blood grouping serum of rabbit origin was dissolved in a minimal (17 ml) amount of low titer type A plasma previously absorbed with type B red cells Anti B blood grouping plasma was prepared in a similar manner Serial dilutions of the anti A blood grouping plasma caused a trace of agglutination of type A red cells to a titer of 1 128 Similar tests of the anti B blood grouping plasma showed a trace of agglutination of type B red cells at a titer of 1 256

The naturally occurring agglutinations which involved an incompatibility with respect to the type A blood group were tested for inhibition by specific polysaccharide blood group A reagent[3] in the following manner One drop of specific polysaccharide was added to 0.4 ml of the type O or type B plasma which was to be tested This was incubated for 30 minutes at 25 before being added to the leukocyte suspension in the test for leukocyte agglutinins A positive control test for leukocyte agglutinins contained in the type O or type B plasma was always run A second control consisted of 1 drop of specific polysaccharide blood group A reagent being incubated with the type O or type B plasma before addition of a 2% suspension of type A red cells in saline

The ability of plasma to cause erythrophagocytosis in the presence of incompatible red cells was tested as follows Red cells which were incompatible with respect to the ABO group of the plasma being tested were washed three times in large quantities of physiological saline One half milliliter of a 2% suspension of these red cells was centrifuged and the supernatant was discarded One half milliliter of a leukocyte suspension in plasma collected in the usual manner was added to the sedimented red cells These were resuspended by gentle agitation and incubated for 2 hours at 37 After resettling for 1 hour smears were prepared from the sediment These smears were stained with Wright's stain and examined for erythrophagocytosis

Hemolysins were tested for in the following manner The serum to be

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Blood grouping sera anti A (rabbit) No 2428 14 and anti B No 2429 15 kindly supplied by Lederle Laboratories

[3] Kindly furnished by Sharpe and Dohme Co

be tested was inactivated at 56 for 30 minutes after which 0 5 ml portions of serial twofold dilutions in 0 85% sodium chloride were added to each of nine tubes Normal saline was added to the tenth tube which served as a control One tenth milliliter of fresh normal unheated AB serum (diluted with an equal part of saline) and 0 5 ml of a 3% saline suspension of thrice washed test cells were added to each of the ten tubes which were then shaken The mixtures were placed in a water bath at 37 for 30 minutes centrifuged for 1 minute at 1500 r p m and examined for the presence of hemolysis

To test the potency of the specific polysaccharide blood group A reagent equal volumes of this substance were added to the serial dilutions of anti A blood grouping plasma and incubated for 30 minutes at 25 before the type A test cells were added The specific polysaccharide reagent was found to inhibit the anti A blood grouping plasma at a titer of 1 8

## Results

The method for preparing the leukocyte suspension was simple and produced cells which were viable and did not tend to clump spontaneously The main disadvantage of the technique was that Sequestrene bound the divalent cations and in this way inhibited the action of complement (9)

In all we have performed over 750 tests for leukocyte agglutination on various combinations of normal plasmas and leukocyte suspensions In general the reproducibility of tests on any one day was good but it was poor in most tests from day to day This lack of reproducibility on different days made it difficult to differentiate false positives from true agglutination reactions Usually nonspecific clumping produced a stringy irregular type of clump The clump caused by a specific agglutinin was more tightly packed and symmetrical Occasionally however they were difficult to differentiate morphologically Although each leukocyte suspension was examined for signs of spontaneous clumping one could not be sure that the cells in an individual test had become injured and were clumped nonspecifically Because of this rather rigid criteria were adopted and only those tests which were positive on two occasions were defined as positive for leukocyte agglutination

## Incidence and Distribution

Table I shows the results of tests on the plasma of 24 nonimmunized (i e nulliparous not previously transfused) donors against the leukocytes of 8 normal donors In all 160 combinations were tested

Of these 160 combinations 84 were compatible with respect to the A B and O blood groups and 76 were incompatible There were 30 positive tests In 6 of the 30 tests (20%) the plasma samples and leukocyte donors were compatible with respect to the A B and O red cell

TABLE I

THE DISTRIBUTION OF NATURAL LEUKOCYTE AGGLUTININS

(The blank spaces denote no test The O denotes a negative test The + denotes a positive test )

CELL DONORS

| PLASMA DONORS | TYPE | I O+ | II O+ | III A+ | IV A+ | V A+ | VI B+ | VII B+ |
|---|---|---|---|---|---|---|---|---|
| 1 | O+ | O | O | O | O | O | O | O |
| 2 | O+ | O | O | O | O | O | + | + |
| 3 | O− | O | O | + | + | + | + | O |
| 4 | O+ | O | O | O | O | O | + | O |
| 5 | O+ | O | O | O | O | + | O | O |
| 6 | O+ | O | O | + | O | O | O | O |
| 7 | O+ | O | O | O | O | + | O | O |
| 8 | O+ | O | O | + | + | + | + | O |
| 9 | O+ | | | + | + | + | | |
| 10 | O+ | | | + | + | + | + | |
| 11 | A+ | O | O | O | O | O | O | O |
| 12 | A+ | O | O | O | O | O | O | O |
| 13 | A+ | O | O | O | O | O | O | O |
| 14 | A+ | + | O | O | O | O | O | O |
| 15 | A+ | O | O | O | O | O | O | O |
| 16 | A+ | O | O | O | O | O | O | O |
| 17 | A+ | | O | O | O | O | O | O |
| 18 | A+ | + | O | O | O | O | O | O |
| 19 | A+ | O | O | O | O | O | O | O |
| 20 | B+ | O | O | O | O | O | O | O |
| 21 | B+ | O | O | O | O | O | O | O |
| 22 | B+ | O | O | + | + | + | O | O |
| 23 | AB+ | O | O | O | O | O | + | O |
| 24 | AB+ | O | O | + | O | O | + | O |

types In 24 of the 30 positive tests (80%) the combinations were incompatible The clumps in the compatible reactions were composed entirely of white cells The clumps in the incompatible reactions were a mixture of red and white cells

#### Erythrophagocytosis

When stained smears were made of the leukocytes used in the test for agglutination it was noted that erythrophagocytosis was present in some In the 6 positive tests in which the plasma donors and leukocyte donors were compatible with respect to the A B and O red cell types none

showed erythrophagocytosis Of the 24 positive tests which were incompatible 22 (92%) showed a significant erythrophagocytosis None of the 66 tests which were incompatible with respect to the A B and O red cell types but did not show clumping had a significant erythrophagocytosis Significant erythrophagocytosis was not observed in the absence of agglutination Figure 1 summarizes these data

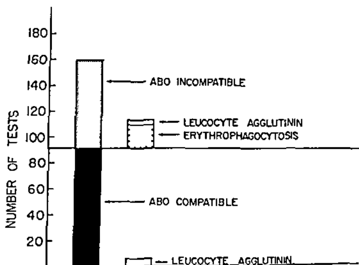



FIG 1 Relationship of the ABO system and leukocyte agglutinins to erythrophagocytosis

## Hemagglutinins

In order to study the role of the anti A agglutinins in the causation of both agglutination and erythrophagocytosis specific polysaccharide of blood group A was added to the test plasma before it was mixed with the leukocytes In 9 of 15 positive tests in which there was an incompatibility due to anti A this inhibition test was performed In all those tested both agglutination and erythrophagocytosis were prevented

## Complement and Hemolysin

Bonnin and Schwartz (2) had previously demonstrated that a hemolysin and complement are required in addition to a hemagglutinin for erythrophagocytosis to occur owing to the presence of an incompatibility for the A B and O red cell types

Complementary action in this system seemed doubtful because of the presence of Sequestrene It was not possible to test this hypothesis di

rectly by heat inactivation however as the white cell suspensions were badly damaged by heating to 56 for 30 minutes

The sera of 6 out of 8 type O plasma donors which produced agglutination and erythrophagocytosis were tested for the presence of hemolysins In only one of those tested was a hemolysin demonstrated

TABLE II

RELATIONSHIP OF HEMAGGLUTINATION ERYTHROPHAGOCYTOSIS AND LEUKAGGLUTINATION

(E P indicates the number of erythrophagocytes found in counting 500 white blood cells The numbers in the columns anti A and anti B are the reciprocals of the titers Otherwise the nomenclature is the same as in Table I )

| PLASMA DONORS | | ANTI | ANTI | | LEUCOCYTE DONORS III | IV | V | VI | VII |
|---|---|---|---|---|---|---|---|---|---|
| | | A | B | EP | A+ | A+ | A+ | B+ | B+ |
| 8 | O+ | 64 | 32 | 269 | + | + | + | + | 0 |
| 3 | O+ | 256 | 128 | 100 | + | + | + | + | 0 |
| 22 | B+ | 256 | | 112 | + | + | + | 0 | 0 |
| 2 | O+ | 256 | 128 | 24 | 0 | 0 | 0 | + | + |
| 5 | O+ | 256 | 128 | 53 | 0 | 0 | + | 0 | 0 |
| 6 | O+ | 128 | 128 | 21 | + | 0 | 0 | 0 | 0 |
| 7 | O+ | 64 | 128 | 37 | 0 | 0 | + | 0 | 0 |
| 1 | O+ | 64 | 64 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | A+ | | 512 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13 | A+ | | 32 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | A+ | | 128 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | A+ | | 256 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | A+ | | 64 | 62 | 0 | 0 | 0 | 0 | 0 |
| 20 | B+ | 64 | | 0 | 0 | 0 | 0 | 0 | 0 |
| 21 | B+ | 64 | | 0 | 0 | 0 | 0 | 0 | 0 |

### Leukocyte Agglutinin

These observations made the usual mechanism for erythrophagocytosis seem unlikely For a time we entertained the idea that the leukocyte agglutinin which always seemed to be present in conjunction with erythrophagocytosis might be important in the etiology of the latter To test this the following experiment was designed Leukocyte suspensions were prepared from blood drawn from the plasma donors This was incubated with washed incompatible red blood cells This test contained plasma donors white cells and plasma in addition to incompatible red cells and the possibility of a leukocyte agglutinin being present was excluded The results are shown in Table II There was a fairly good correlation between the ability of a plasma to cause erythrophagocytosis and mixed red and white cell clumps There is one plasma which causes

erythrophagocytosis but does not cause agglutination There is no explanation for this This suggests the possibility that the mixed cell clumps were caused by an extension of the process of erythrophagocytosis A white cell agglutinin does not appear to cause this mixed cell type of clump

Cinephotomicrographic studies of the process of the formation of a mixed cell clump lend some support to this contention When the leukocyte suspension and incompatible red cells are mixed as described above the red cells become spherocytes and clump Next after about 15 minutes the white cells become sticky and coming in contact with the spherocytes become attached to them Then the white cells engulf the red cells by surrounding them with pseudopodia This is a complicated process and may take 15 to 30 minutes Eventually the red cells are taken into the white cells Subsequently two erythrophagocytes join The film strip (Fig 2) shows that this occurs in a specific manner The chance touching of a white cell pseudopod to the red cell pole of the erythrophagocyte causes sticking of the two It appears that the red cells are attractive to another erythrophagocyte even though they are inside the white cells

It is not clear why some plasmas have a stronger tendency to cause erythrophagocytosis than others This will require further study

## Pure White Cell Clumps

On the other hand the clumps which were made up entirely of white cells in the tests involving compatible plasma and white cell donors seemed to be caused by a true agglutinin The naturally occurring agglutinins were uniformly weak and difficult to reproduce but behaved much like leukocyte agglutinins which we have found in typing sera of rabbit origin

In 1928 Wichels and Lampe (15) observed that white cells from two patients of blood group A with myeloid leukemia could be agglutinated by anti A agglutinins These cells also were capable of absorbing the anti A agglutinin Thomsen (13) in 1930 repeated these experiments with 8 leukemic patients and found only occasional agglutination reactions He did demonstrate absorption of the corresponding agglutinins by leukocytes Twible *et al* (14) by the use of combined cross matching and absorption techniques demonstrated the A and B antigens in human leukocytes The antigen could be shown in only 15% of the population by direct cross matching and required absorption methods for demonstration in the remainder Dausset (4) using powerful typing serum which also contained a hemolysin and using care to

get a fairly pure white cell suspension was able to demonstrate the A and B antigens in white cells In addition he showed the lack of agglutination after absorption with A or B red cells or inhibition with Group A and B substances (Witebsky s substance) Likewise Bus (11)

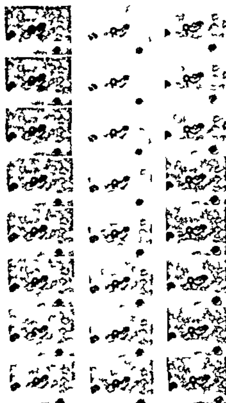


FIG 2 Film strip showing two erythrophagocytes joining The first strip shows the brush borders of the leukocytes in contact In the second strip the brush border of the leukocyte on the right is moving upward toward the red cell pole of the leukocyte on the left In the third strip it finally makes firm union with the red cell pole of the leukocyte on the left

more recently has tested white cell suspensions against anti A and anti B sera of human origin He reported that white cells behaved in the same manner as the corresponding red cells

When anti A and anti B blood grouping plasmas were substituted for the test plasma in the test for leukocyte agglutinins the results illustrated in Table III were obtained Both plasmas were effective in caus

ing clumping to about the same extent against white cells from donors of types O A B and AB Note the prozone effect

When specific polysaccharide was added in sufficient quantity to neutralize the anti A agglutinin the agglutination of these white cells was essentially unaltered (Table III) These results indicate that this anti

TABLE III

ACGLUTINATION OF HUMAN LEUKOCYTES BY BLOOD-GROUPING PLASMA (RABBIT)

(The — denotes a negative test + to ++++ denotes various degrees of a positive test the blank spaces denote no test When only three dilutions were tested the typing plasma was neutralized by the addition of specific polysaccharide of blood group A )

| LEUCOCYTE DONOR TYPE | ANT A | | | | | | | | | | B | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 2 | 1 4 | 1 8 | 1 16 | 1 | 1 64 | 1 2 | 1 2 | 1 51 | nd | 1 2 | 1 | 1 8 | 1 16 | 1 32 | 1 64 | 1 128 | 1 2 | 1 5 2 |
| O | | | | | | | | | | | | | | | | | | | | |
| O | | | | | | | | | | | | | | | | | | | | |
| O | | | | | | | | | | | | | | | | | | | | |
| O | | | | | | | | | | | | | | | | | | | | |
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| B | | | | | | | | | | | | | | | | | | | | |
| B | | | | | | | | | | | | | | | | | | | | |
| B | | | | | | | | | | | | | | | | | | | | |

serum contains a potent leukocyte agglutinin unrelated to the A B and O red cell groups Further studies are required to determine the origin of this agglutinin It is unlikely that it is of rabbit origin as other experiments indicate that normal rabbit serum contains agglutinins only to a titer of 1 4 against human white cells

## Summary and Conclusions

The plasma of 24 normal donors was tested against the white cells of 7 normal donors In all 160 combinations were tested and 30 showed agglutination reactions Two types of cell clumps were noted—the "pure white cell clump and the mixed red and white cell clump The mixed cell clump was the result of testing plasma against white cell suspensions from donors who were incompatible with respect to the A B and O blood groups The mixed cell type of clump appears to be the result of a strong tendency on the part of certain plasmas to cause erythrophagocytosis rather than the action of a leukocyte agglutinin

Some studies are presented which concern a leukocyte agglutinin found in blood grouping plasma of rabbit origin This leukocyte agglutinin is not related to the A B and O blood groups

## REFERENCES


1 Ackroyd J F *Progr Allergy* **3** 531 (1952)
2 Bonnin J A and Schwartz L *Blood* **9** 773 (1954)
3 Boorman K E Dodd B E and Loutit J F *Lancet* **1** 812 (1946)
4 Dausset J *Compt rend soc biol* **148** 1607 (1954)
5 Dausset J Nenna A and Brecy H *Blood* **9** 696 (1954)
6 Goudsmit R and van Loghem J J Jr *Vox Sanguinis* **3** 3 (1953)
7 Harrington W J Sprague C C Minnich V Moore C V Aulvin R C and Dubach R *Ann Internal Med* **38** 433 (1953)
8 Landsteiner K *Wien klin Wochschr* **14** 1132 (1901)
9 Mayer M M Croft C C and Gray M M *J Exptl Med* **88** 427 (1948)
10 Moeschlin S and Schmid E *Acta Haematol* **11** 241 (1954)
11 Rus P *Acta Haematol* **14** 30[illegible] (1955)
12 Stefanini M and Plitman G *J Clin Invest* **32** 606 (1953)
13 Thomsen O *Acta Pathol Microbiol Scand* **7** 250 (1930)
14 Twible E A Tullis J L and Diamond L K in Blood Cells and Plasma Proteins (J L Tullis ed) Academic Press New York 1953
15 Wichels P and Lampe W *Klin Wochschr* **7** 1741 (1928)

# Added Comment

J L TULLIS
Harvard University Boston Massachusetts

Knowledge of the existence of leukocyte antibodies and leukocyte antigenicity dates back many decades in France to the early work of Bordet and in this country to the work of a distinguished physician who is with us at this Symposium As an effulgent young house officer at the Boston City Hospital thirty years ago Dr Charles Doan made a study of transfusion reactions within compatible red cell groups and he clearly demonstrated by supravital techniques the presence of potent antileukocyte agglutinins which sometimes agglutinated the white cells of a recipient after transfusion with group specific blood

More recently (1949 1950) both the investigators with whom Dr Finch has been associated and another group working in Boston enthusiastically entered the field and rediscovered the presence of iso and immune white cell antibodies When it was realized that these factors had been described previously little extension of that work was made other than to rename the leukocyte antibody *leukotoxin* in deference to Dr Doan s original nomenclature The subject then was temporarily put aside until about two years ago when our group reopened a study of the characterization and quantitation of leukocytic agglutinins

Meantime in Europe some very classic and provocative work was in progress Dr Sven Moeschlin carried out both *in vitro* and *in vivo* experimentation on the antigen antibody relationships of pyramidine induced agranulocytosis Utilizing the Harrington type of transfusion experiment he produced severe leukopenia in a normal recipient by the transfusion of serum from a sensitized donor

At approximately the same time Dr Marcel Bessis clearly delineated by time lapse photomicrography a series of morphologic changes which characterized the degeneration of a leukocyte after exposure to its natural antibody Dr Bessis in association with Drs Andre and Dreyfus also reported a case of lymphatic leukemia occurring in a person with Hodgkin s disease who had been splenectomized because of leukopenia

As a result of his study he postulated "La leukopénie et la thrombopénie sont expliquées par la présence d'un anticorps antileukocyte et anti plaquette"

Concerning the work just presented the findings of both Dr Finch and Dr Butler have clearly broadened our thinking on the role which leukocyte antibodies may play in both pathologic and so called normal reactions

Time precludes a discussion of the iso antibodies but good evidence has been presented that leukocytes do possess the standard A B and O blood groups as well as others as Dr Finch and Dr Butler have shown

In the area of the immune antibodies the evidence points to a significant role of leukocyte factors in the natural course of lymphomas and leukemias especially the aleukemic variety but as yet no firm proof has been adduced to show any *etiologic* role for these antibodies in malignant disease

Work on such a possibility nonetheless goes forward with great enthusiasm and vigor especially by Dr Max Seligman at the Pasteur Institute Paris I have not seen a publication of his work but it is my impression that he has been able to demonstrate three distinct antigens in normal leukocytes by employing the agar technique

Our eagerness to interpret such concepts of antileukocyte antibodies as regulatory mechanisms for the numbers of circulating leukocytes should not deter the pursuit of other and perhaps equally important humoral factors however For example why should the total colligative properties of serum from leukopenic individuals show an inordinate lessening of the freezing point depression when contrasted with non leukopenic controls? And why in a series of twenty eight recent leukopenics from whom we were able to isolate leukocyte agglutinins should the total electrolyte and crystalloid particles of the serum be reduced to an average of 294 milliosmoles compared with a normal of 312 in a corresponding group of leukopenics in whom no leukocyte agglutinins could be isolated? Does this mean that an anti white cell antibody has the ability to bind an excess of salt at the temperature at which water freezes? Certainly it would be surprising if such levels of extracellular hypotonicity actually existed in the intact organism

These questions must await further study Meanwhile it is safe to conclude that the leukocyte antibodies probably are true antibodies and protein in type

Table I shows that a quantitation of leukotoxin is possible by a simple pipet technique The average decrease in leukocyte count in an *in vitro*

mixture of normal white cells and normal type specific sera is only 2% at the end of 30 minutes and 4% at the end of 60 minutes

Table II for comparison shows the decrease in count through agglutination and lysis which follows a similar *in vitro* mixture of normal leuko

TABLE I
NORMAL SERA IN LEUKOTOXIN TEST

| Number of tests | Range at 30 minutes % | Range at 60 minutes % | Average % | |
|---|---|---|---|---|
| | | | 30 minutes | 60 minutes |
| Negative 52 | 0–10 | 0–14 | 2 | 4 |

TABLE 2
SERA CONTAINING ANTI WHITE CELL BODIES IN LEUKOTOXIN TEST

| Number of tests | Range at 30 minutes % | Range at 60 minutes % | Average % | |
|---|---|---|---|---|
| | | | 30 minutes | 60 minutes |
| 28 | 8–70 | 12–79 | 30 | 40 |

TABLE III
SPECIFICITY OF LEUKOCYTE ANTIBODY FACTOR
(Two positive sera A S and J S )

| Serum | 0 | 30 minutes | 60 minutes |
|---|---|---|---|
| A S | 33 500 | 30 100 (−10) | 28 400 (−15) |
| J A | 20 900 | 15 300 (−27) | 13 600 (−35) |
| 0 25 ml same sera + 0 25 ml concentrated leukocytes 18 hours at 4 | | | |
| Serum | 0 | 30 minutes | 60 minutes |
| A S | 32 400 | 32 200 (0) | 29 900 (−7) |
| J A | 21 300 | 20 900 (−2) | 20,200 (−5) |

cytes and abnormal sera In this series of 28 positive sera there was an average decrease of 30% at the end of 30 minutes incubation and 40% at the end of 60 minutes

Table III shows the specificity of the reaction wherein it is possible to adsorb the antileukocyte activity by exposure to an excess of leukocytes and thus reverse previously positive tests to negative tests

Finally, is the antileukocyte factor truly a protein and if so what kind of protein is it? Figure 1 shows the location of the antileukocyte activity in paper strip electrophoretic analysis of abnormal sera. In this figure the paper strip is represented schematically on the abscissa and the antibody activity of the different protein fractions eluted from various segments of the strip is plotted on the ordinate. It may be seen that the

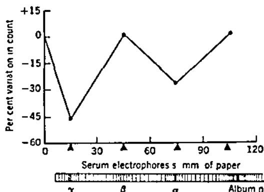



FIG 1 Electrophoretic location of antileukocyte activity

major concentration of antileukocyte activity is in the portion of the electrophoresis corresponding to the gamma globulin moiety with no activity in the albumin or beta globulins. Why a moderate amount of antileukocyte activity was found in the alpha range is unknown.

In summary, on the basis of the work presented today plus supporting investigations from both the present and the past, a few conclusions may be presented:

1 Leukocyte antibodies are factors of clinical importance.

2 They play an important role in the natural history of an occasional case of lymphoma and an occasional case of aleukemic leukemia.

3 These factors apparently are proteins with characteristics similar to other gamma globulins.

# General Discussion

DR THEODORE S HAUSCHKA (Buffalo New York) I should like to point out a refinement in technique applicable to the study of iso-antigens of leukocytes Using inbred strains of mice Amos [*Brit J Exptl Pathol* 34 464 (1953)] was able to produce antibodies directed against specific gene controlled histocompatibility factors Titers up to 1 in 512 are common and titers up to 1 in 2000 have been reached The technique has been used to demonstrate circulating antibodies present after skin grafting between mice of different strains [*Brit J Exptl Pathol* 35 203 (1954)]

DR JACOB FURTH (Boston Massachusetts) Dr Tullis mentioned the presence of antileukocyte antibodies in the lymphomas Would you like to elaborate on that? Does that have anything to do with the etiology of the disease or is it one of the manifestations of the disease or complications such as occur in agranulocytosis? Is there any specificity in those antibodies in their action between normal lymphocytes or malignant lymphocytes?

DR J L TULLIS I do not believe there is any relationship etiologically between the genesis of Hodgkin s disease or lymphoma and the presence of auto-antibodies Out of our group of twenty eight patients with leukocyte agglutinins reported there were three cases in which the underlying abnormality was Hodgkin s disease All three were *suffering from leukopenia* with a presumptive diagnosis of hypersplenism secondary to activity in the spleen The remainder of the group of twenty eight were predominantly made up of persons with so-called primary hypersplenism

I was interested in hearing that aminopyridine is the most common agent in Dr Moeschlin s entire group Most of our leukocyte agglutinins were from people with cirrhosis of the liver or portal vein thrombosis

DR ROGER ROSSITER (*London Ontario Canada*) *I should like to make a few* remarks on Dr Finch s most excellent paper on the production of antisera with different fractions of leukocytes from exudates of the guinea pig

We have fractionated exudate cells on a number of occasions not from the guinea pig but from the rabbit by the usual process of differential centrifugation in sucrose and our experience with the various fractions has been quite disappointing We find that when we do chemical determinations on the various fractions at least 60% of the leukocyte RNA is in our nuclear fraction

On examining this nuclear fraction we find that probably the reason for this is that in the multilobed nucleus of polymorphs one has very large chunks of cytoplasm complete with mitochondria included in the nuclear fragment For this reason I don t think it is surprising that Dr Finch finds that with antisera from his nuclear fractions he does obtain quite marked activity Most of his activity certainly is in the cytoplasmic fractions but there is some in the nuclear fraction

We are quite puzzled and should very much like to develop good techniques for obtaining nuclei from the polymorphonuclear cells I certainly should like to talk with Dr Finch about this and with anyone else who has had experience with differential centrifugation of polymorphs

DR S C FINCH We have had no experience studying the nuclear protein content of these fractions but by morphologic observation *it is true that one always* certainly has considerable amounts of cytoplasmic fragments attached to the nuclei We have felt that possibly this may have been responsible for some of the anti

leukocytic activity that we have had with nuclear antisera I think we can be reasonably sure however that our cytoplasmic fractions contained very few nuclei

I should like to mention one other set of essentially negative experiments which we have completed These were attempts to produce autoleukocyte antibody by isoimmune mechanisms In this study we took a number of guinea pigs made peritoneal exudates removed the spleens and bone marrow mixed each of them with Freund's adjuvant and administered them separately to normal guinea pigs subcutaneously and intraperitoneally We used both nuclear and cytoplasmic fractions *and did complement fixation studies to try to detect the presence of any leukocyte* antibody in the normal recipients as well as multiple peripheral blood counts I do not think we obtained any consistent findings in terms of either depression of white cells or evidence of complement fixation In other words we were completely unsuccessful with the possible exception of perhaps some slight activity with the cytoplasmic fraction of the granulocytes

Dr Albert S Gordon (New York New York) I should like to direct a question to Dr Finch

First of all do these agglutinins act peripherally on the circulating leukocytes?

In view of Dr Craddock's work showing the rapid regenerative capacity of the leukocytes after leukopheresis is there any possibility that these agglutinins act on stem cells within the marrow?

*If the action is peripheral what happens to these leukocytes that are agglutinated?*

Dr S C Finch Those are some very embarrassing questions to which I do not have the answer I think we can say at the present time only that the white cells disappear Other studies done by Dr John Lawrence a number of years ago and by Dr Cajano more recently have demonstrated that there is an early depression or depletion of the bone marrow elements and later there is a very active and rapid regeneration These studies suggested that the transient depression of bone marrow elements more or less corresponded to the period of peripheral leukopenia

We do not know where these white cells go We assume they are badly damaged and rapidly removed from the circulation but we do not know whether or not they are sequestered and whether or not they enter the circulation again later

Dr Wolf W Zuelzer (Detroit Michigan) I should like to ask Dr Butler a question

In ruling out the effect of anti A or anti B do you consider the neutralization of the A or B by specific soluble substance effective? I wonder if you have taken into account the possibility of immune antibodies against A or B because they are not readily neutralized by A or B substances

Dr J J Butler I think that is a possibility although we ran controls on these with red cells and there was no effect of the neutralized anti A on the red cell *control*

Part II

# Genetic and Environmental Factors in the Transmission of Leukemia

Chairman
Howard R Bierman
City of Hope Med cal Cente
Duarte California

# 5

# The Pathophysiology and Immunogenetics of Transplantable Leukemia

THEODORE S HAUSCHKA and JACOB FURTH
Roswell Park Memorial Institute Buffalo New York and
Children s Cancer Research Foundation Boston Massachusetts

Recent advances in the biology of experimental leukemia have broadened the basic knowledge prerequisite to an effective clinical approach Activity in the field may be inferred from the large number of reviews since 1950 dealing with chemotherapy histopathology hormonal aspects and etiology (25 27-30 38 39 53 83 85 88 102 105 139 141) Because the complex variables inherent in the general laboratory use of grafted tumors are often poorly understood and the observations interpreted incorrectly the present survey is focused on the pathophysiology and immunogenetics of the transplantable leukoses

Leukemia represents a heterogeneous group of growth disorders resulting either from changes in the cells ability to respond to host forces which normally regulate growth and maturation or from a derangement of these forces The significance of chemotherapeutic "cure" in rodents has been exaggerated it should be remembered that leukemic grafts and the animals bearing them are not exact analogs of either the growths or the hosts in spontaneous disease

Factors in the etiology of leukemia to which transplantation studies have contributed include especially antigenic mutations and transmission attempts by filterable agents or cell fragments In the tempting but unreliable extrapolation of data from transplantable leukemias to causative factors four independent categories of gene action should be appreciated the heritable influence in "spontaneous" leukemias the genes promoting susceptibility to chemical and physical carcinogens the mutational steps of "progression in established tumors and finally the immunogenetic aspects of "take"

The latter two are significant sources of variation in graft behavior If primary genetic differences exist between neoplastic and corresponding normal cells they are too remote and too confused by subsequent cellular changes to be divulged through analysis of serially transferred tissue for example the pathophysiology of a transplanted granulocytic leukemia or of an ascites lymphoma affords no clues to the original anomalies which led to their induction

Preoccupation with pathologic and genetic features of advanced neoplasia has perhaps distracted investigators from the potentially rewarding search for ephemeral impairment of white cell "homeostasis and for possible dependent precursors of autonomous leukemias (41)

## Pathophysiology

### Transplantable Tumors of the Hemopoietic System

The first well studied transplantable leukemia was described by Snijders in the guinea pig (31) After many earlier attempts to transmit rodent leukoses in heterogeneous animals Korteweg (99) carried an induced mouse lymphosarcoma through 17 consecutive transfers obtaining up to 44% takes Since then most types of leukemia have been established in serial passage

There are close analogies between these growths and the corresponding lesions in man (30 31) Lymphocytic granulocytic monocytic mast cell and plasma cell leukemias as well as reticulum cell neoplasms resembling Hodgkin s disease are listed in the comprehensive catalog of Dunham and Stewart (28) It is doubtful however if the precise equivalents of small cell lymphoid leukemia chronic granulocytic leukemia acute leukemia of childhood Hodgkin s and multiple myeloma of man have been reproduced in animals

Besides the thirty eight mouse tumors listed by Dunham and Stewart eleven transfer lines in the rat and one guinea pig lymphoma are described Rodent leukemias have a much greater diversity than those of the domestic fowl which occur in a few monotonously uniform kinds and are transmissible with cell free agents Most leukemias of the lower mammals are of the acute lymphocytic type the frequency of granulocytic and other forms roughly parallels the normal differential white cell count (Table I)

Experimental leukoses can be grown as systemic tumors by intravenous transfers Intramuscular injection of the same cells usually produces solid tumors which can be carried as such Since the various neoplasms of the hemopoietic system have responded well to experimental conversion into the ascitic form—i e they can grow as cell suspensions

in peritoneal exudate (49 50 90 91)—ascites tumors have considerable advantages over their solid counterparts for quantitative studies on the growth spread cytology and biochemistry of malignant tissue

TABLE I
AVERAGE NUMBERS OF THE DIFFERENT LEUKOCYTES PER 100 WHITE CELLS AND FREQUENCY OF CORRESPONDING LEUKEMIAS

| Cell type | Mouse | Rat | Man |
|---|---|---|---|
| Neutrophils | 21 | 25 | 70 |
| Eosinophils | 3 | 2 | 2 |
| Basophils | Rare | Rare | Rare |
| Lymphocytes | 68 | 67 | 24 |
| Monocytes | 6 | 5 | 3 |
| *Leukemia frequency* | | | |
| Myeloid | ~15% | ~17% | ~45% |
| Lymphatic | ~70% | ~70% | ~37% |
| Monocytic | ? | ? | ~ 9% |

* The differential counts are based on the monograph of Gardner (45) the leukemia frequencies in man are statistically analyzed in two recent papers by Gilliam (48) and Hewitt (77)

Table II lists twenty three ascites leukemias useful for cytologic and cell population analysis

TRANSPLANTABILITY

On the whole lymphocytic leukemias are rigid in their host requirements and can be grafted only to animals of the inbred strain in which they originated Host survival time is reduced during successive serial passages until it reaches a constant low level Individual transfer lines often have characteristic distinguishing features the same applies to granulocytic leukemias except for somewhat lesser virulence Transplantable reticulum cell sarcomas described in several strains of mice and rats represent a heterogeneous group One extreme is related to monocytes (macrophages) the detached circulating member of the reticuloendothelial system the other extreme is the fixed reticuloendothelial cell The former can be considered as an aleukemic form of monocytic leukemia Some of these produce on transplantation pleocellular tumors resembling Hodgkin s disease The latter cannot always be distinguished with certainty from granulomatous lesions Dunn (30) who studied reticulum cell neoplasms recently describes three morphologic types The neoplastic character of type C is very doubtful types A and B are slow growing and transplantation of B rarely succeeds even

TABLE II
TRANSPLANTABLE LYMPHOMAS AND LEUKEMIAS GROWING AS ASCITES TUMORS

| Tumor | Home strain | Origin | Ascitic *transfer* generation | Usual *color of* exudate | Modal ploidy | AR and AD lines available | Established in the ascites form by |
|---|---|---|---|---|---|---|---|
| 6C3HED lymphosarcoma | C3H/St | Chem | 243 (R) | B | s | No | Klein 1951 (90) |
| 6C3HED adapted to DBA/2 | C3H/St | Chem | 9 (R) | W | 2s | No | Hauschka *et al* 1956 (75) |
| DBA/2 lymphoma (thymus) | DBA/2 | Spont | 221 (R) | W P | s | No | Klein 1951 (90) |
| PLS lymphosarcoma | AKM | — | — | — | — | No | Klein 1951 (90) |
| A#1 lymphoma | A/He | X ray | 152 (R) | W P | 2s | No | Shelton 1954 (151) |
| A#2 lymphoma | A/He | X ray | 186 (R) | B | s | No | Shelton 1954 (151) |
| E L 4 lymphoblastic leukemia | C57BL | Chem | ~200 | W | — | No | Gorer 1950 (58) |
| AK4 leukemia | AK | Spont | — | — | — | No | Goldie 1956 (49) |
| P1534 lymphatic leukemia | DBA/2 | Spont | — | — | — | No | Goldie 1956 (49) |
| C1498 granulocytic leukemia | C57BL/6 | Spont | — | — | — | No | Goldie 1956 (49) |
| DL1 lymphoma | DBA/2Ha | Spont | 9 (R) | P W | s | No | Amos (unpublished) |
| E L 24 lymphatic leukemia | C57BL | Chem | 4 (R) | W | s 2s | No | Bock (unpublished) |
| KL1 lymphoma | C58 | Spont | 10 (R) | P | — | No | Amos (unpublished) |
| L1210 lymphocytic leukemia | DBA/2 | Chem | 73 (N) | B | — | Yes | Law and Potter (unpublished) |
| L4946 lymphoma (thymus) | AKR | Spont | 85 (N) | W | — | Yes | Law and Potter (unpublished) |
| L5178 lymphocytic leukemia | DBA/2 | *Chem* | 38 (N) | P | — | *Yes* | Law and Potter (unpublished) |
| P288 lymph node leukemia | DBAf/2 | Chem | 50 (N) | B | 2s | No | Law and Potter (unpublished) |
| P335 lymphocytic leukemia | DBAf/2 | Chem | 42 (N) | B | — | No | Law and Potter (unpublished) |
| P433 lymphoma (thymus) | DBAf/2 | X ray | 21 (N) | B | — | No | Law and Potter (unpublished) |
| P228 reticulum cell type A | DBA/2 | Chem | 18 (N) | P | — | No | Law and Potter (unpublished) |
| H5530 granulocytic leukemia | C58 | Spont | 11 (N) | B | — | No | Law and Potter (unpublished) |
| 70429 plasma cell leukemia | C3Hf/He | Chem | 28 (N) | W | — | Yes | Law and Potter (unpublished) |
| Murphy lymphosarcoma | Wistar rat | Chem | 45 (R) | W P | — | No | Day *et al* (unpublished) |

*Symbols* Chem = chemically induced B = bloody P = slightly bloody W = blood free s = near diploid 2s = near tetraploid AR and AD = antimetabolite resistant or dependent lines The ascitic transfer generations are current serial passages at the Roswell Park Memorial Institute Buffalo (R) or at the National Cancer Institute Bethesda (N)

in the inbred strain of origin Type A also may regress It appears therefore that some of these could be dependent neoplasms or if autonomous they are not fully compatible with the hosts on which they were grafted Some may not be neoplasms at all

The highly specific histocompatibility of mouse leukemias is exemplified by uniform "take" of tumor C1498 in all mice of the C57BL/6 line and equally regular regression in an isogenic subline which arose by mutation of a single H factor (18 19) Even in the immunologically neutral anterior eye chambers lymphoma homografts (i e interstrain grafts as distinguished from isografts within the strain of origin) grew poorly and heterografts not at all (119) Primary grafts often have a very long (up to one year) and variable latent period Transplantation sites and other host factors may influence the percentage and character of takes In our experience uniformity begins after about the second subpassage

GROWTH

Diffuse leukemias would appear quite unsuitable for exact growth measurements The increment of certain ascites tumors has been plotted however by Revesz and Klein (144) who used quantitative rinsing and dye dilution procedures Three lymphomas of different anatomic and genetic origin showed increments of similar order Cell number in the peritoneal cavity increased exponentially during the most active growth phase and attained a maximum of about 2 billion per mouse The accumulation of exudate was not directly proportional to cell number Cell concentration per unit volume of fluid reached its peak earlier than total number in the peritoneal cavity and decreased progressively during later stages of growth as did the volume of individual cells

Doubling rate i e cell generation time for a given inoculum of the E L 4 DBA/2 and 6C3HED lymphomas varied between 5 and 10 hours and seemed correlated with mean cell volume This suggestive relationship between cell size and doubling time depends on limitations by the host as apparent from the growth behavior of a near diploid ascites carcinoma and its tetraploid subline (74) The cells of the latter have twice the diploid volume but multiply at the diploid rate during the early logarithmic phase until the total cell mass—drawing on the host s nutritional and other resources—becomes the limiting factor The end mass attained and survival time were the same for both tumors the mice succumbed after supporting the synthesis of about 2 billion diploid cells or 1 billion tetraploid cells

Exact growth measurements for ascites lymphomas are handicapped

by two principal sources of error Infiltration of viscera by the malignant lymphocytes begins early and morphologic distinction between the malignant and some inflammatory cells by the criteria of exfoliative cytology is difficult (95) Fortunately most ascitic lymphomas are fairly pure cultures of the inoculated cell type The admixture of normal host exudate cells amounts to about 5% (93) Existing information on the free cells of normal peritoneal fluid and on their response to various stimuli (32 144) allows realistic corrections in cases of uncertain morphology

The cytologic analysis by Felix and Dalton (32) of native cells in peritoneal fluid confirms and extends earlier observations Cellular response to injection of 0 85% NaCl melanin granules or carbon particles followed the same general sequence An immediate rise in neutrophilic granulocytes was succeeded by a slow continuous numerical increase of macrophages whereas lymphocytes decreased initially and then became more numerous Activity of macrophages was at times accompanied by a considerable amount of mitosis (up to 1%) Transformation of lymphocytes to macrophages was claimed but should be documented by time lapse cinematography The above findings describe the reaction to intraperitoneal stimuli The situation is more complex in the ascites leukemias where hemopoietic organs are infiltrated by the tumor cells here the character of the exudate depends both on the material in the peritoneum and its inroads on hemopoietic structures of the host

The ascites lymphoma growth determinations (144) serve as useful models for the course of acute autonomous disease They furnish a norm for judging experimental growth inhibition by antimetabolites antibody or irradiation and provide physiologically more revealing reference data than would tumor weight host weight or survival time

## Virulence

The term virulence designates the rapidity of evolution and fatality of the leukemic process In the immunogenetic literature virulent has been used synonymously with nonspecific" to denote increased host range because genetic indifference is among the progressive changes observed during prolonged serial transfer (78 153) However extremely virulent neoplasms with long transplantation histories may retain rigid iso antigenic specificity for ten or more years (e g the diploid Gardner lymphosarcoma 6C3HED)

The cause of fatality is complex in all malignant neoplasms One may point to cachexia anemia hemorrhage infection or infiltration of vital organs as lethal factors

Leukemic mice are seldom cachectic although the leukemic cells may

function as "metabolic traps" of nitrogen and lipids Infection is rare in mouse leukemia Anemia and hemorrhage are common in some strains but are seldom the immediate lethal factors Thymic tumors are often associated with hemorrhagic pleurocardial effusion causing compression of the lungs Grafted leukemias even of thymic origin do not cause such massive thymic involvement Death of rodents bearing trans planted leukemia may result most frequently from the invasion of vital organs by neoplastic leukocytes

Virulence can be altered experimentally by temporary serial transfer through incompatible animals (72) The induced decline in growth po tency can manifest itself as a decreased take percentage in the normally 100% susceptible home strain and has been interpreted by Mitchison as a "function of length of residence in the foreign strain" (130 131) Hoecker and associates (80) have described reduction of takes for several trans plantable mouse leukemias after a side passage of only 7 days The period of growth in refractory hosts necessary to damage 6C3HED lym phosarcoma irreversibly is about 10 days (131) When the same tumor was transferred every week through 84 consecutive passages in the re sistant Swiss mice it survived for 20 months but it died out shortly after the transfer period was increased to 10 days (75)

Useful data on virulence have accumulated for the Gardner lympho sarcoma The observations cover over 200 weekly passages in the sus ceptible C3H strain of origin followed by three years of continuous passage in refractory Swiss and DBA/2 mice and terminated with 9 serial passages after return to the C3H home strain The survival times of over 1000 mice corresponding to the above transplantation history were 11 4 ± 0 1 days for C3H mice bearing the virulent tumor and 79 2 ± 3 8 days for C3H mice inoculated with the subline attenuated by passage through Swiss When the latter tumor was maintained in C3H for 9 consecutive transfers survival time gradually dropped from 79 to about 20 days (67 75 and unpublished data of Hauschka *et al* ) During the prolonged sojourn in the C3H home strain the nuclear mor phology of the lymphosarcoma shifted spontaneously from spherical to lobated (113) after transfer to the foreign strains the formerly always hemorrhagic exudate became practically colorless and invasion of the viscera was not evident Invasiveness and hemorrhage need not be re lated however Leukocytes do move in and out of vessels Ability to stimulate capillary formation or interference with factors of hemostasis may be influential

The least virulent yet about 90% lethal transplantable tumor on record is a DBA adapted tetraploid subline of the 6C3HED lymphosarcoma

(75 *and unpublished observations*) which requires an average 7 2 months (range 3 to 16 months) to kill its hosts DBA/2 mice inoculated with this ascites tumor develop enormous ascites (average 23 6 ml) without serious debility Metastasis is infrequent The exudate abounds in viable lymphoma cells a stable mitotic index of about 1% is compensated for by an efficient balance between tumor growth and cell death

INVASIVENESS

Malignant white cells like normal leukocytes are migratory but they are more invasive Their aggressive infiltrations show histologic preferences *characteristic of the transplantable strain*

Although injections of cell free plasma from leukemic rodents have given negative results in normal animals cellular portions of the blood reproduce the disease (42 43 134) The amount of blood required to transmit the Lewis rat lymphoma #8 only 1 day after subcutaneous implantation *is exceedingly small* Lewis *carried this tumor through* 12 serial passages by means of whole blood (116)

The invasiveness of mouse ascites lymphomas (6C3HED A#2 DBA/2 E L 4) begins during the first day of intraperitoneal growth (144) On the fifth day they are invariably transmissible with 0 1 ml of peripheral blood diluted 1 200 taken from the tail tip or from the retro-orbital sinus (75 and Kaziwara unpublished observations) Granulocytic mouse leukemia C1498 provoked an accumulation of serous exudate after intraperitoneal inoculation whence it rapidly invaded blood and hemopoietic organs (51) The hemograms in the ascites and in peripheral blood were parallel (52)

Observations of Furth and his associates linking lymphosarcoma with leukemia are confirmed by the effect of implantation site on the disease pattern *of the Murphy lymphosarcoma in Wistar rats When the tumor* was transferred intraperitoneally blood lymph nodes and thymus were involved Localized lymphosarcoma extending only as far as the regional lymph nodes developed after subcutaneous grafting (134) A similar role of inoculation site as a factor in metastatic behavior was found by Goldie *et al* (52)

The invasive character of a lymphoma can change spontaneously and can also be altered experimentally Mouse tumor 6C3HED estrogen induced by Gardner *et al in* 1941 *was not metastatic during its first* 25 transplant generations despite its rapid growth (46) Later its metastatic capacity became pronounced and is now a permanent feature of its diploid main line—yet very rare in a tetraploid subline derived by immunoselection in refractory hosts (75)

A concomitant change in number of mitochondria and increased invasiveness in a lymphoid leukemia during serial transfer suggest that metastatic cells differ genetically from the less autonomous elements in the primary growth (127) The pattern of leukemic spread in hybrid mice is less uniform than in animals of pure strain (82) Furth and his collaborators had shown earlier that the spread of neoplastic white cells is retarded by immunogenetic resistance AK leukemias take only in a small percentage of RF mice where tumors form at the site of intramuscular injection whereas in AKs they spread freely and produce the generalized disease Preirradiation of the hosts facilitates dissemination

Among the most intriguing aspects of leukemic spread are the preferential tissue affinities which distinguish certain morphologically similar transfer lines from each other (30) For example one of five cell strains studied by Furth *et al* (43) had a specific ovarian tropism enlargement of the ovaries being in some cases the only sign of "take Strain *RG10* produced growths in the kidneys whereas line *S27* invaded the muscles by preference These muscular infiltrations were sometimes massive spread about the spinal cord and penetrated nerves and ganglia with consequent paralysis of the hind legs Three of five strains studied never produced pleural and peritoneal effusions but *S27* evoked a hemorrhagic and *Ar* a milky white ascites Pulmonary involvement invasion of pancreas enlargement or pericapsular infiltration of the lymph nodes and perivascular or diffuse invasion of the liver varied sufficiently from one lymphoma strain to another to be almost diagnostic These histopathologic findings certainly indicate the importance of "soil as a factor accessory to passive mechanical cell distribution in tumor spread (166) The nature of these affinities (antigenic humoral or other?) is unknown and offers fertile opportunities for future research

It is thus apparent that metastatic behavior of neoplastic grafts depends on a multiplicity of factors including host genotype antigenicity and chromosome constitution of the tumor cells cell size and motility tissue preferences and inoculation dose or site

DEPENDENCE ON HOST FACTORS

Early attempts to characterize leukemias as true neoplasms such as the successful transplantation of single leukemic cells (44) focused interest on autonomy Practically all the transplantable mammalian leukemias overwhelm their hosts through unrestrained exponential proliferation It is a challenging thought that "benign" conditioned hemoblastosis might precede the malignant emancipation from control by the host A systematic search for dependent mouse leukemias—aided by growing in

formation on life span and maturation stages of normal and malignant leukocytes (139)—may yield valuable research material This expectation is warranted by two examples (1) AK spontaneous leukemias were generally transplantable within the AK stock but several chronic myeloid leukemias arising in RF mice could not be serially propagated in the inbred strain of origin (41) (2) The tetraploid subline of the 6C3HED ascites lymphoma referred to above kills female DBA/2 mice but regresses in males After several males had apparently recovered from the tumor they were injected with estradiolbenzoate (the same hormone with which Gardner had induced the 6C3HED lymphosarcoma fifteen years earlier) The ascites promptly reappeared and killed them The original diploid tumor growing in *C3H* mice is much too virulent to reveal a subtle hormonal dependence but the tetraploid variant wages a seesaw battle with its DBA hosts lasting up to 16 months and behaves like a conditioned neoplasm (Hauschka and Grinnell unpublished observations) The observations of Dunn (30) on nontransplantable reticulum cell sarcomas may also be cited in this connection

*As for the nature of leukopoietic regulators nothing definite is known* The existence of a feedback type of regulation can be postulated This may resemble the highly specific dependence of grafted thyroid cells on the presence of thyrotropic hormone Some hormonal regulators may be less specific e g the degree of somatotropic influence from the pituitary Still others could be nutritional as shown by growth inhibition of lymphosarcoma grafts in pyridoxine and riboflavin deficient mice (159 160)

The hypothesis that the crucial change in leukemia resides in the host s impaired filtration and destruction mechanisms (14) was not strengthened by the filtration experiments of Ambrus and associates (2) In the latter work isolated lungs of C57BL/6 and DBA/2 mice carrying transplanted leukemias C1498 or P1534 were as efficient in leukocyte removal as control lungs Conversely leukemic and nonleukemic leukocytes were removed by the lungs of normal mice at identical rate and were later released from the heart lung preparations when white cell free blood was introduced Additional evidence is needed however for the filtering capacity of lungs from mice with spontaneous leukemia Such experiments now in progress have not revealed any significant difference (C M Ambrus *personal communication*)

In a speculative discussion Weiss (165) has presented clues to the self limiting character of growth His own work and the cited observations of Danchakoff Willier and Ebert indicate that "compounds produced by a given cell type have some selective effect on the same cell

type and that this homologous effect is instrumental in the regulation of growth" For example extra embryonic insertions of spleen grafts or minute liver fragments produced corresponding enlargement of spleen and liver in chick embryos (165)

Is the total mass of the normal leukocyte population similarly regulated by white cell growth products or through more indirect humoral control according to the "needs of the host"? Granulocyte secretions have been described and filmed by Richter (145) host response to these and less obvious secretory products is a possibility

A vast body of data supports the influence of endocrine factors on the production tissue distribution and disposal of white blood elements (53) Estrogens and the pituitary adrenal axis are among the most potent stimuli (39 40) It has been emphasized by Kaplan (85) and others (13) that in mice the thymus is the fulcrum through the stimulation or involution of which most hormones exert their enhancing or retarding power on leukemogenesis Changes in the differential leukocyte count in female mice have been correlated with the estrus cycle (10) *A striking sex difference in the total weight of organoid lymphoid tissue* (9) and greater susceptibility of female mice to lymphatic leukemia (120) are on record This relationship is not uniformly true however In the RF strain thymectomy prevents lymphoma induction by x rays but not induction of myeloid leukemia the incidence of which is increased *the latter because thymectomy prevents death of many mice from thymic* lymphomas (Upton and Furth to be published) In $LAF_1$ mice the leukemia induction rate is greatly enhanced in females by ovariectomy (Buffett and Furth to be published)

Endocrine responses are not limited to normal hemopoiesis and initiation of leukemia (46) *but are exhibited by transplantable autonomous* lymphomas as well Although adrenalectomy increased susceptibility to rat lymphosarcoma grafts (161) the same operation inhibited the development of transplanted lymphatic leukemia in mice (Silberberg *et al* as quoted by Kaplan 85) Chromosome size in mouse leukemic cells was altered by sex hormones (16) A sex discrepancy (takes in females were twice those in males) in the acceptance of DBA/2 thymic lymphoma *grafts by test hybrids could not be altered by castration* (68 76)

All gradations may conceivably exist between leukemia with fully host dependent cells and leukemia with autonomous cells Although the usual course of events during serial transplantation leads toward emancipation from growth restraining influences changes in experimental leukemia need not always be *progressive shifts toward increased malignancy* Decreases in virulence (75) increases in antigenicity (68) and sus

pected reversion to hormonal dependence (Hauschka and Grinnell unpublished observations) have recently been induced in mouse lymphomas by incompatible passage immunoselection and other experimental means The mechanism involved here is probably selection of mutant cell types responsive to temporarily altered factors in foreign host genotypes and is thus similar to the selection of antimetabolite dependent mutants in Law s experiments (102 104 106)

## Immunogenetics and Related Cytology

### HISTOCOMPATIBILITY

Serial transplantation of leukemias tends to create a fluctuating genetic gap between graft and host strain An appreciation of this variable should moderate the assessment of chemotherapeutic and other results often obtained under conditions of imperfect compatibility Dependence of histocompatibility on the presence in the host genotype of certain dominant hereditary factors in graft and host was first postulated by Little 42 years ago Methods for the analysis of immunogenetic differences have been consolidated through the transplantation experiments of Strong and Bittner the serological work of Gorer and Snell s elaborations of allelism and linkage Law s enlightening review (105) traces this classical chapter of mammalian genetics from its beginnings to its latter day complexities There can be no doubt that the gene controlled iso antigenicity of the cell surface plays a decisive role in the fate of both normal and malignant homografts including leukemic transplants

If a tumor graft carries one or more dominant loci functioning in the elaboration of antigens and the recipient has immunologically different alleles of these factors the implant evokes antibodies and usually regresses The most fully analyzed and "strongest" locus among several histocompatibility factors is H 2 represented by at least ten phenotypes in various inbred mouse strains (153 155)

Recent refinements in the genetic localization of the antigens controlling tumor transplantation include evidence for autosomal linkage (60 105 155) mapping of H 2 on the ninth chromosome (1) crossing over—i e pseudo allelism—within the apparently composite H 2 "locus" (1 6) X and Y linkage (results of Strong Bittner Eichwald and own results discussed by Hauschka 70 76) The detailed characterization of specific H factors and corresponding antigens has become possible through the combined use of linked marker genes isogenic resistant mouse strains (153) and iso agglutinating test sera produced in the latter (see below)

Hereditary aspects of leukemia compatibility were first analyzed for

leukemia line I by MacDowell and Richter who reported a one gene difference between the C58 strain of origin and resistant STOLI mice (123) Their data are based on the conventional backcross test in which the Mendelian percentages of tumor deaths reveal the underlying genetic segregation The analysis was carried one crucial step further into a second backcross" generation which essentially confirmed the one gene difference and at the same time hinted at the participation of minor as yet unidentified factors

Pursuing their discovery that certain C57BL/10 individuals were incompatible for granulocytic leukemia C1498 which originated in this strain and which had in the past been uniformly lethal for over 200 serial passages Borges and Kvedar (18) discovered an H mutation in the mouse This was identified as a change from H 2b to H 2d (19 154) Progressive growth and regressions of leukemia C1498 in resistant backcross and F hybrids were in excellent agreement with a one gene difference between the original line and its isogenic resistant offshoot now called C57BL/10-X

The quantitative merits of ascites lymphomas in transplantation genetics are clear from recent work in several laboratories (5 7 8 59 67 75 92) The transplantation behavior of the ascites lymphomas propagated by serial transfer of intraperitoneal cell suspensions is stable over long periods Stroma necrosis and infection—important variables in solid tumor grafts—are less apt to complicate ascites growth The agreement between Mendelian expectation and actual lethal takes" is usually very close as is evident from tests with four ascites lymphomas summarized in Table III

The DBA/2 lymphoma gave an interesting *site specific* result (Table III lines 1 and 2) Four or five H factors were needed for progressive subcutaneous growth in backcross mice whereas intraperitoneal growth required only two genes This raises the question whether antigenicity could be affected by tissue specific differences between the grafted tumor and host stroma or other cells in close physical contact with the implant

Subcutaneous regressions have also been observed for the 6C3HED lymphosarcoma in susceptible C3H mice (146) In unpublished experiments of Amos after subcutaneous implants of 6C3HED ascites had regressed in C3H/St mice these animals should have been immune to intraperitoneal challenge given 1 to 3 months later but they were unexpectedly susceptible On the other hand occasional subcutaneous regressions of the L#2 lymphoma ascites (A/He origin) in the related A/St strain conferred the usual immunity to subsequent intraperitoneal

TABLE III

HISTOCOMPATIBILITY TESTS WITH FOUR ASCITES LYMPHOMAS OF THE MOUSE IN RESISTANT BACKCROSS (RBC) AND F HYBRIDS

| Ascites lymphoma | Strain of origin | Test hybrid | Total mice inoculated | Per cent lethal takes | Per cent expectations | H factors required for take |
|---|---|---|---|---|---|---|
| DBA/2 lymphoma | DBA/2 | (DBA/2 × C3H) RBC | 200 i p | 23 | 25 | 2 |
| | | | 114 s c | 5 | 6 | 4? |
| | | (DBA/2 × C3H) F | 207 i p | 55 | 56 | 2 |
| 6C3HED lymphosarcoma | C3H/St | (C3H × DBA/2) RBC | 331 i p | 53 | 50 | 1 |
| | | | 328 s c | 41 | 50 | 1? |
| Lymphoma #1 | A/He | (C3H × A) RBC | 76 i p | 58 | 50 | 1 |
| | | | 76 s c | 53 | 50 | 1 |
| | | (C3H × A) F | 70 i p | 81 | 75 | 1 |
| | | | 77 s c | 73 | 75 | 1 |
| Lymphoma #2 | A/He | (C3H × A) RBC | 128 i p | 50 | 50 | 1 |
| | | | 126 s c | 46 | 50 | 1 |
| | | (C3H × A) F | 79 i p | 79 | 75 | 1 |
| | | | 80 s c | 77 | 75 | 1 |

Summarized from data of Hauschka (68) Hauschka *et al* (75) and Hauschka and Grinnell (unpublished observations)

challenge The distinct reactivity of the subcutaneous site compared to intraperitoneal response is not a general phenomenon however as may be seen from data in lines 6 to 13 Table III (Hauschka unpublished observations)

The histocompatibility of the DBA/2 lymphoma was also subject to a probably X linked influence which first became apparent by a diminished number of takes among the backcross males Since castration did not correct this deficiency X linkage was investigated The frequency of takes was found to depend on the presence ($F_1$♀ × C3H♂ = BC type 1) or absence (C3H♀ × $F_1$♂ = BC type 2) of one X chromosome from the susceptible DBA/2 grandparent in the two classes of reciprocal backcross hybrids of the genotype ($C3H_f$/He × DBA/2) × $C3H_f$/He Takes for BC type 1 were 26% in 236 males and 38% in 270 females for BC type 2 18% in 207 males and 28% in 208 females This X influence like the inhibitory role of the subcutaneous site is peculiar to the DBA/2 ascites tumor for it was not found in three other lymphomas similarly tested (76)

Not all the genetic determinants of graft survival need be cellular antigens of the blood group variety Although the H 2 complex and other histocompatibility loci function through immunological mechanisms metabolic traits or hormonal levels under genetic control by which the inbred donor strain differs from the recipient may also manifest themselves as graft dependence Such humoral or nutritional needs of the graft could on occasion mimic the segregation of iso antigens in test hybrids Blocking merely the immune response of a host by radiation or cortisone gives therefore no assurance that the metabolic requirements of the "successful" homograft or heterograft are adequately met

SEROLOGY AND PROTECTIVE IMMUNIZATION

Gorer (55) gave an immunologic slant to the genetic basis of transplantation and was first to investigate the role of antibodies in host reactions to transplanted mouse leukemia (56) The complex H 2 "blood group" was characterized both genetically and serologically as the principal locus of mice active in tissue immunity (60) Histocompatibility 2 determines several cellular antigens at once—a situation comparable to the *Rh* or *MNS* systems in man (65 153)

The earlier rather course saline hemagglutination method (54) has been refined to a dextran-enhanced procedure Iso antibody can now be determined regularly at dilutions up to 1 4000 and not infrequently to 1 16000 (59 61) Agglutination tests have been extended by Amos

to leukocytes with an iso antibody against lymphogenous mouse leukemia E L 4 (3)

These advances in serological technique coupled with the production of antisera in Snell s isogenic resistant lines (153) prepared the way for a thorough analysis of the eight or more distinct antigenic factors controlled by the H 2 system (6 7 79) The elaboration of this system now facilitates crucial studies—not only of antigenic simplification but also of "new antigens in malignant leukocytes

According to MacDowell s earlier work (122) a single intraperitoneal injection of fetal skin embryonic viscera or adult liver from strain STOLI (but not from strain C58) conditioned C58 mice to resist an otherwise lethal challenge of C58 line I leukemia Inoculation with STOLI tissue also rendered C58 mice antagonistic to four additional transplanted lines of C58 leukemia (M liv M kid A L) but was ineffective against lines S T and U and against first transfers of fourteen spontaneous cases

Since the latter grew at best much more slowly than the virulent line I it is apparent that the establishment of immunity does not hinge on the degree of virulence Generally those leukemic lines capable of inducing resistance to themselves were also cross reactive with STOLI tissue Acquired immunity could not prevent the later onset of spontaneous leukemia in the treated C58 mice experimental immunization apparently becomes effective in proportion to the cumulative genetic gap resulting from long term serial transfer

In similar tests Brncic *et al* (23 24) achieved complete or partial protection against an AK leukemic line in AK mice which had survived a lethal dose of C58 leukemic cells and in C58 mice pretreated with AK neoplasms The two transplantable leukemias used H from AK and A from C58 produced a generalized leukemia in nonimmune hosts and killed them in a few days Partially protected animals had atypical localized lymphosarcomas and survived significantly longer Protection also followed prior treatment with homologous leukemic material inactivated by hypotonic saline (34)

Considering the fact that antigenic identity between graft and host is questionable in some instances where effective immunization against transplanted leukemic cells was obtained the available data are nevertheless instructive Among the more recent cases are reports of induced resistance of C3H mice against their "own 6C3HED lymphosarcoma (35 36)

Those experiments in which antigenic material and hosts were judiciously controlled according to genotype permit the inference that the

antibodies operative in transplantation immunity are not as a rule specifically antineoplastic but are influenced to a large extent by isoantigenic relationships (65) For example the *in vitro* neutralizing power of an antiserum for A strain leukemic cells was not weakened by absorbing the saline hemagglutinins From this Gorer (56) concluded that mice also produce nonagglutinating protective antibodies The latter could be absorbed by leukemic cells and to a much lesser extent by red cells Despite the quantitative absorption differences the two types of antibody appeared to be directed against the same iso antigen

Passive transfer of acquired antileukemic resistance by means of cells was originally accomplished through intraperitoneal injection of minced liver from immunized mice which was followed at once by a challenging tumor dose (122, 142)

Brncic *et al* (23) transferred protection from resistant to susceptible mice by transplanting spleen thymus and lymph nodes liver kidney brain or skeletal muscle had no effect These authors also investigated the dosage aspects of passive transfer (unpublished data) Fifty milligrams of immune isologous spleen was the minimal protective weight Significantly less immune tissue was required when homologous leukemic material was tested for instance AK leukemia transplanted into C58 Passive transfer was abrogated by treating the immune AK donors with cortisone before introducing their spleen pulp into susceptible AK mice (47)

In his work on passive transfer of transplantation immunity against lymphosarcoma 6C3HED Mitchison (130 131) demonstrated protection only after transfer of local lymph nodes—not however with corresponding nodes from the side opposite to the tumor inoculation site Neither spleen nor muscle from immunized mice was effective Failure of spleen probably reflects the local inoculation route employed rather than an inherent inability to protect Since immune lymph node mince invariably failed to produce tumors in susceptible CBA mice Mitchison expressed the opinion that resistance was genuinely passive and not the consequence of contamination with viable tumor cells The possible transfer of fragmented or nonviable antigenic material has not been excluded however Passive immunity remained firm for 4 days became weak after 10 days and was lost after 20 days

In all the above experiments living cells donated by immunized animals were essential for the passive transfer of protection The reaction whereby normal guinea pig serum is highly effective against 6C3HED lymphoma cells *in vivo* but not *in vitro* suggests host participation through a mechanism not fully understood (86 163) Heterologous

to leukocytes with an iso antibody against lymphogenous mouse leukemia E L 4 (3)

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antisera produced by rabbits against the Murphy rat lymphosarcoma (137) or against the 6C3HED mouse lymphoma (138) destroyed the corresponding tumors *in vivo*—a step beyond inactivation *in vitro* (56 148)

Passive *in vivo* protection by cell free iso antiserum presents a more difficult problem which was successfully attacked by Gorer and Amos (4 62) In this work they took advantage of the extreme sensitivity of C57BL leukemia E L 4 to anti H 2 sera A strain C3H and BALB/C mice were hyperimmunized against E L 4 until their sera showed high hemagglutinating activity When such sera were injected into non immunized mice of foreign strains the usual temporary growths of E L 4 did not develop at the inoculation site unless at least a week had elapsed between serum treatment and tumor challenge Subsequent tumor im plants produced a typical primary response indicating that this was true passive immunity

Either E L 4 cells or normal C57BL liver were able to remove the hemagglutinating activity from the anti E L 4 sera however the pro tective activity could be canceled only by absorption with E L 4 leu kemic cells not with normal C57BL tissues This interesting result sug gests that besides the H 2 component the serum contained a second antibody against an unknown antigen called "X Anti X after removal *of anti H 2B by repeated absorptions with normal C57BL tissue* gave potent protection against the C57BL ascites leukemia even in C57BL mice X may therefore be regarded as a good antigen" (59)

Similar immunity has been obtained against three other lymphomas (6C3HED DBA/2 A#2) protection with corresponding antisera being characterized by a delay in tumor development in homologous and isolo gous animals The antisera against these lymphomas were fractionated into two components one of which is precipitable at 13 5% sodium sul fate and the other at 18% The former fractions which were largely gamma globulin contained all the hemagglutinating activity but did not inhibit tumor growth in mice of the home strain the 18% fraction was protective and was therefore assumed to be directed against a "tumor specific" entity similar to the X in E L 4 (8)

There are at least three possible explanations for this previously un known system of antigens (*1*) H mutations in neoplastic leukocytes (*2*) H mutations in the host strains (*3*) viruses on which the cells have come to depend Histocompatibility mutants among the tumor cells (involving qualitative change rather than loss) should theoretically be incompatible and hence would have little competitive value in a com patible cell population It also is improbable that each of four different

inbred host strains (C57BL C3H DBA/2 and A) has undergone the same general type and degree of antigenic alteration resulting in X Therefore the change seems to reside in the leukemic cells

The apparent frequency of "X antigens" in mouse leukemias as well as the many data on auto immunization in hemological diseases (33 37 135) prompt the speculation that malignant leukocytes may be weakly antigenic in their original hosts Thus leukoblastic lysates of acute leukemia patients showed two but lacked one of the main anti genic components of normal leukocytes they also contained a new particular antigen different from any of those in normal leukocytes (150) If there is a response to these antigens it would perhaps do no more than clear the blood stream of the neoplastic cells or localize the tumor at a given site The presence or absence of such hypothetical auto antibodies may account for the difference between leukemic and so called aleukemic leukemias or between lymphosarcoma and generalized lymphatic leukemia

## Chromosome Balance and Graft Compatibility

The nucleocytoplasmic ratios in leukocytes make metaphase spreading difficult and are responsible for the relative scarcity of quantitative chromosome data Biesele s measurements (15 16) of chromosome size in mice established the following relationships The chromosomes were largest in long established transplantable leukemia and embryonic lymphocytes intermediate in the lymphocytes of spontaneous disease and smallest in the normal lymphoblasts of adult mice The size differ ences were ascribed to amounts of pepsin digestible protein rather than DNA

Nuclear lobation and fragmentation was followed through the entire mitotic cycle in the Gardner lymphosarcoma ascites (113) The karyo meres often referred to as "rosette" nuclei by pathologists undergo syn chronized prophases The metaphase chromosomes form a normal plate anaphase is regular and lobation is re established during the nuclear swelling of late telophase

Improvements in squash techniques such as the aceto orcein method have made it possible to obtain accurate counts of chromosome num bers especially in the ascites lymphomas (71 112 113 151) About two thirds of these leukemic cell populations are near diploid several interesting near tetraploid strains are on record The latter include A strain lymphoma #1 the DBA/2 adapted line of the Gardner lympho sarcoma (75) and Potter s DBA/2 lymph node leukemia P288 (Table II)

Determinations of deoxyribose and ribose nucleic acids likewise have shown a predominance of diploid values (91 125) However elevated DNA and RNA phosphorus was encountered by Menten *et al* (126) in measurements of splenic lymphocytes from leukemic mice as compared with normal spleens

In a series of integrated cytologic and genetic studies a general relationship between chromosome constitution and transplantation specificity was established Diploid tumors transplanted within the species but outside the iso antigenic barriers set by the genotype of origin fail to grow progressively Aberrations of the chromosome number (heteroploidy of varying degrees) favor more or less indiscriminate violations of immunogenetic boundaries (67 68 71 73 146 147)

This correlation applies as yet without exception to a representative histologic variety of tumors including several mouse lymphomas Confirmatory serologic tests (5 75 81) outlined in Table IV show a significant weakening of antigens as a consequence of polyploidization The essential feature of heteroploidy in so far as it affects gene controlled iso antigenicity of the cell is not chromosome doubling as such but genic imbalance Neither point mutation in a histocompatibility locus nor physical loss of chromosomes carrying important H factors need be involved in bringing about antigenic surface changes Since the molecular configurations of the glycolipoprotein cell surface are probably controlled by a variety of genes—antigenic ones as well as others—any change in the total dosage relationship of loci to each other may displace or alter molecules with specific antigenic function located in the cell membrane It is through surface recognition that the host accepts or rejects the graft

The principle of interrelationship between chromosome number and histocompatibility was put to a crucial test by exposing the diploid 6C3HED lymphosarcoma ascites which always contains a small number of polyploid cells to the selective pressure of immunologically hostile environments (75) During prolonged transfer through refractory mice a near tetraploid subline of the tumor was isolated This was able to kill the formerly refractory DBA/2 mice although it was much less virulent and invasive than the original diploid ascites The iso antigenic specificity of the polyploid derivative was reduced to feeble hemagglutinin absorbing titers in comparison with the potent diploid 6C3HED material A small residue of diploid lymphoma cells giving an H 2k reaction persisted in the tetraploid ascites during its two year absence from the C3H home strain It was possible to reselect this minority gradually toward predominance in the neoplastic population by re

TABLE IV

CORRELATION BETWEEN CHROMOSOME NUMBER ISO-ANTIGENICITY AND HOST SPECIFICITY IN SEVERAL MOUSE LYMPHOMAS

| Ascites lymphoma | Modal chromosome number | Strain genotype | Iso-antigens of strain of origin | Iso-antigens of tumor | Transplantation specificity | Known violation of H-2 barriers |
|---|---|---|---|---|---|---|
| A#2 lymphoma | 44 | H-2a | CDEFK | CDEFK | Specific | None |
| A#1 lymphoma | ~80 | H-2a | CDEFK | Weak CD<br>Almost absent EFK | Not specific | H-2d |
| 6C3HED | 40 | H-2k | CdEfK | CEK | Specific | None |
| 6C3HED DBA/2 adapted | ~80 | H-2k | CdEfK | Almost absent | Not specific | H-2d |
| 6C3HED Swiss adapted | ~46 | H-2k | CdEfK | Almost absent | Not specific | Several unrelated strains |
| DBA/2 lymphoma | 42 | H-2d | CDE$^d$Fk | CDE$^d$F | Specific | None |
| DBA/2 lymphoma Swiss adapted subline | ~85 | H-2d | CDE$^d$Fk | Not tested | Not specific | Several unrelated strains |

Summarized from data of Hauschka and Levan (71) Amos (5) Hoecker and Hauschka (81) and Koprowski (97 98 and unpublished observations)

turning the tumor to compatible C3H animals Thus immunoselection of neoplastic leukocytes both away from and back to the H 2 specific diploid genotype can be experimentally controlled through the use of appropriate hosts

EXPERIMENTAL ALTERATIONS OF GRAFT RESPONSE

Although the mechanism underlying the genetic indifference of heteroploid grafts can be interpreted as *immunoselection other means of* altering host resistance or graft response are less well understood (68 164) Among these methods are irradiation cortisone treatment adaptive immunization foster nursing of the host and specificity changes induced in the graft after intermittent growth in hybrids or through egg passage

Total body irradiation of refractory mice utilized by Furth *et al* (43) abrogated resistance to leukemic implants Preparatory radiation treatment also permitted generalized infiltration of liver and spleen in Sprague Dawley rats cross circulated with a noncompatible chloroleukemia from Sherman rats (J Hollingsworth unpublished observations)

Heterografts of human myeloid lymphatic and paramyeloblastic leukemia persisted and grew for a while in rats blocked by cortisone and nitrogen mustard (21) However promising this and similar successes may appear the physiological incompatibility of the unnatural environment extends beyond the temporarily eliminated antigenic barriers Conclusions drawn from the reactivity of such leukemic implants to chemotherapeutic and other agents are therefore less reliable than data gained from iso or homografts

Law (100 101) has demonstrated a susceptibility" influence present in the milk of DBA/2 foster mothers during the first week of the lactation period Refractory DBA/1 and C3H mice nursed by such mothers supported the growth of lymphoid leukemias LL449 and P1534 A single passage through foster nursed individuals permitted these two lymphomas to grow in serial transfer in resistant hosts giving the same clinical picture as in the home strain After reinoculation into the susceptible strain the leukemic cells lost their temporary nonspecificity which indicates that the milk influence must arise from within the refractory mice No such factor was evident in similar tests with myeloid leukemia LL493

Another quite unrelated foster nursing effect known as the maternal resistance factor (MRF) is contributed by old foster mothers from certain low leukemic strains This milk factor does not apparently in

fluence the course of transplantable leukemias (124) although it over rides the genetic predisposition toward a high incidence of spontaneous leukemia (102)

Numerous injections of refractory C57L mice with lyophilized C57BL myeloid leukemia C1498 prior to challenge with fresh tumor could not produce the anticipated enhancing phenomenon" commonly obtained with other neoplasms On the contrary the leukemic homoiotransplants failed to grow even temporarily (152) The large conditioning dosage of dry frozen tumor should have made the C57L hosts tolerant (84) similar to repeated grafting of living cells (115) These contradictory results may be ascribed to differences of pretreatment dosage and of immunogenetic traits in host and graft they need not indicate the existence of separate enhancing and inhibitory substances especially since both effects may be achieved with the same tumor and both are removable by filtration

The principle of actively acquired tolerance" to foreign skin (17) has been confirmed with several strain specific mouse lymphomas (97 98 and Koprowski personal communication) Blood isologous with the malignant leukocytes was injected into embryos of a refractory strain Mice so treated during fetal life succumbed in adult life to the otherwise incompatible lymphomas 6C3HED L#2 and DBA/2 After a single passage through mice conditioned in utero subsequent serial transfer in untreated individuals of the foreign strain became possible Thus secondary changes in the population of graft cells (immunoselection?) appear to be superimposed on the initial "adaptive" alteration of host immunity

An interesting change in tumor host relationship can be brought about by serial passage of the DBA/2 lymphoma and other neoplasms through the chorio allantoic membrane of the incubating hen s egg (128 129) After one egg passage the lymphoma became permanently compatible with DBA/1 mice it also took for two or more successive transfers in about 40% of Swiss C57BL/6 Albino Marsh and Bagg mice Egg adaptation did not permit growth in C3H mice from which the tumor differs by two histocompatibility genes The egg adapted ascites growing in the foreign strains showed no significant changes in modal chromosome number incidence of polyploidy or nuclear lobation it contained however numerous macrophages neutrophils and pyknotic nuclei typifying a continuing immune reaction Mere superficial coating of the malignant lymphocytes with egg substance after magnetic stirring at 4 did not weaken their antigenic potency It therefore appears that during growth in embryonated eggs (and perhaps also in other nonspecific

media) the tumor cell surfaces incorporate environmental constituents which temporarily neutralize the mouse iso antigens Thereby they retard antibody mobilization in genetically refractory hosts and allow more time for selection of compatible cells

Histocompatibility of tumor grafts can be permanently modified during a single passage through a hybrid genotype (11 12 66 94) This discovery has been further substantiated by tests with mouse lymphomas (65 66 68 75 158) it appears to involve a general principle Although $F_1$ hybrids between susceptible and resistant strains resemble the susceptible parent in giving 100% tumor takes they are not immunologically neutral $F_1$ infants for example are much more refractory to low cell dosages of the 6C3HED lymphosarcoma than C3H infants (75) Similarly cultured leukemic spleen cells of C58 origin produced leukemia in all inoculated mice of the C58 strain even after 3 months *in vitro* But after only 6 weeks of cultivation transplantability to animals from a cross between C58 males and Bagg albino females was completely lost (87) This probably reflects a decrease in the inoculum a certain tumor cell dosage being necessary to overcome the mild immune response in $F_1$ hybrids Genetic tests performed before and after $F_1$ passage have shown either decreased or increased specificity (11 66 68 94) These induced shifts in antigenicity are usually not haphazard but may proceed from a good fit for a given number of histocompatibility genes to an equally close fit for a smaller or larger gene requirement

Since these changes in transplantation potential become manifest after only brief exposure to $F_1$ influences are irreversible and repeatable and exhibit Mendelian ratios they have fundamental implications in tissue genetics Are they merely instances of immunologic selection or antigenic "adaptations"—or do they hint at true somatic recombination (110)? The latter interpretation is especially challenging but is somewhat dampened by the inability of the $F_1$ effect to obliterate iso antigenic reactivity controlled by the H 2 locus One gene gaps between grafts and backcross hosts identified as the differences between the *H 2 antigens D and K or A and K were unaltered after two consecutive* $F_1$ passages of the 6C3HED lymphosarcoma (75) and the L#2 lymphoma (Hauschka and Kvedar unpublished observations)

## Leukemia Transmission with Cell Fragments and Tissue Filtrates

The initial aim of transplantation with counted numbers of cells was to establish a dose response curve to demonstrate the autonomy of malignant leukocytes and to prove that transmission of leukemia was due to the presence of living" cells in the inoculum Single cells transmitted

the disease but large numbers of mechanically crushed cells failed to do so under the conditions of earlier experiments (44) Subsequently single-cell techniques have been utilized in clonal studies which were primarily concerned with the mosaic nature of neoplastic cell populations and with questions of somatic mutation (67)

Single cell takes in infant mice can be obtained with yields of up to 30% and the ascites growth derived from intraperitoneally injected cells appear within 3 to 4 weeks Claims of tumor transmission with chromatin fractions or tissue centrifugates are therefore subject to the criticism that these preparations contained a few microscopically un detectable viable cells A more portentous interpretation ascribes the resulting tumors to the uptake of chromosome debris Although trans plantation of subcellular genic entities from one mammalian cell to an other is as yet in the realm of fancy this phenomenon is a well estab lished fact in bacterial genetics (109 110) The relevant experiments with leukemia are therefore discussed under the purely speculative common denominator of transduction" with emphasis on the need for more crucial tests in this area

Genetic transduction as defined by Lederberg (109) includes under one term a variety of genic recombination mechanisms Some of these for example karyogamy and heterokaryosis occur in nature others were foreshadowed by the experimental pneumococcus transformation of Griffith the active substance being a specific nucleic acid of the deoxy ribose type (121) The units of exchange are fragments smaller than cells ranging from whole nuclei through karyomeres to minute por tions of chromosomes Pieces lacking kinetochores presumably would align themselves with the host-cell idiogram either by haphazard tandem attachment or by orderly physical replacement of homologous gene regions Transduction is a problem not merely of genetic addition but of functional integration such as the substitutions of allelic flagellar anti gens in *Salmonella* (111) It is tempting to presuppose a similar anti genic exchange in the influence of $F_1$ hybrid hosts on the histocom patibility patterns of transplanted mouse lymphomas (see preceding section)

Klein s experiments with the 6C3HED lymphosarcoma and the DBA/2 lymphoma (92) were intended as a genetic check on tumor transmission achieved with chromatin fractions by Stasney *et al* (156 157) Since the tumors which appeared in Klein s $F_1$ hybrid mice after massive intraperitoneal injection of chromatin were compatible with the susceptible parent strain he concluded that they were not induced *de novo* in $F_1$ tissue but resulted from cell contamination in the nuclear

fraction Although this is an orthodox genetic argument there are occasional exceptions to the general rule that $F_1$ grafts take only in genotypes which carry the essential iso antigens of both parent strains Schweitzer and Furth (149) found that leukemias arising in $F_1$ mice (AK × RF) behaved in transplantation experiments like those originating in AK that is they were transplantable to the AK parental stock and hybrids but not to the RF stock Klein s $F_1$ tumors arising after lymphoma chromatin (in which no cells were microscopically detectable) was administered were almost all solid growths whereas Hauschka always obtained ascites after inoculating only twenty to thirty intact viable cells of the same two tumors Does this perhaps suggest that intimate physical contact between the "transducing" chromatin particles and receptive host cells—such as would occur in the needle path through the body wall but not in the peritoneal fluid—is essential for the conversion of normal lymphocytes into malignant cells?

The difficulty of unequivocal proof for the absence of cells in active *fractions has been faced squarely in the original as well as later experi*ments of St isney *et al* (156 157) and Paschkis *et al* (140) conducted with the Murphy rat lymphosarcoma Within 2 weeks (!) after subcutaneous injection of lymphosarcoma chromatin 33% takes were obtained The localized tumors resembled the Murphy Sturm lymphosarcoma histologically and were accompanied by frequent leukemic infiltrations in liver spleen and kidney There was no leukemic blood involvement The microscopic examination of active chromatin samples revealed no tumor cells When 3000 neoplastic lymphocytes the minimum number found necessary to induce comparable tumor growth were deliberately added to a chromatin suspension and this mixture was recentrifuged cells were easily recognizable in smears It seems improbable therefore that contaminating cells would consistently have escaped detection in fixations from eighty separate experiments and in a painstaking search through serially sectioned chromatin isolated from 45 grams of lymphosarcoma

Leukemic organ infiltration occurred in only 12% of the 202 rats developing local lymphosarcoma after subcutaneous inoculation of several thousand tumor cells—a quantity which would have been detectable in the chromatin fraction *The incidence of invasiveness was much* higher (43% in 44 rats) after subcutaneous injection of tumor chromatin If the latter had contained a few intact cells a different pattern and lower frequency of leukemic lesions might have been expected

Among additional rather convincing though indirect arguments against viable cells as the leukemogenic factor in their work Paschkis

and his collaborators (140) list the following observations Freezing and thawing or exposure to room temperature rendered the chromatin inactive but did not destroy the viability of cell suspensions These are acceptable but debatable arguments since many cells perish at room temperature or during freezing and thawing transmission of leukemia with such materials is usually accomplished by the surviving cells The tumor inducing quality of chromatin in the reported experiments may depend on the intact structure of the chromonema In contrast with lymphosarcoma fractions similar hepatoma material never induced tumors when injected subcutaneously to be effective it had to be introduced intrahepatically This could be interpreted to mean that trans duction" cannot occur in the absence of receptive compatible cell types i e the subcutaneous site contains a sufficient number of hemopoietic cells responsive to lymphosarcoma particles but lacks liver cells with an avidity for hepatoma fragments Implantation of a few tumor cells into the anterior eye chambers of rats was followed by rapid tumor growth but no tumors developed after intra ocular implantation of hepatoma chromatin alone It would be informative to repeat this experiment with lymphosarcoma chromatin for few if any normal lymphocytes are found in the anterior eye chamber (102) although inflammation will promptly bring them to any site

Uptake of tumor chromatin would presuppose phagocytic activity of homologous host cells for which evidence is lacking Since karyomere formation is a cytologic feature of many white cells a mechanism exists for synchronizing the "ingested chromatin with the mitotic sequence The separate micronuclei and lobes undergo simultaneous prophases and their chromosomes enter a bipolar metaphase plate whence they are equally distributed to daughter cells (113) Bits of nuclear material from leukemic lymphocytes incorporated by normal cells during a compatible phase of DNA replication might thus merge with host nuclei and render them malignant

This assumption (140) has been called "the most explicit claim of transduction outside the bacteria" (110) However linkage of the transmitted neoplasia with the donor cells histocompatibility type in Klein s $F_1$ experiment was thought to require a rather large unit of exchange If we admit the genetic basis of malignancy and consider autonomy the end point of a multiple mutation sequence the chance for transposing the malignant constellation together with antigenic markers into one synthetic genome seems quite remote On the other hand cellular emancipation from growth restraining host forces may involve diverse nonspecific nuclear alterations or states of genic imbalance

This is implied by the differences between spontaneous tumors from genetically homogeneous mouse stocks—or even from the same individual mouse—with respect to growth rates and take percentages in test hybrids (118)

Random introduction of chromatin from leukemic donor tissue into the diploid chromosome complement of normal $F_1$ lymphocytes would produce varying degrees of aneuploidy and intrachromosomal rearrangements Among the possible consequences of such genic imbalance are cell death benign metabolic confusion oncogenesis and weakening of the specific iso antigens on the surfaces of the altered $F_1$ cells The latter could thereby attain transplantability beyond the limits of immunogenetic rule (69 71 73 75) Genetic proof for the origin of malignant lymphocytes (in $F_1$ hybrids treated with chromatin fraction or in parental genotype?) is therefore incomplete evidence against the "transduction" of neoplasia with nuclear fragments

The open question of whether the effective unit in the chromatin transmission experiments is cellular or subcellular is paralleled by the equally unsettled problem of leukemia transmission with filtered tumor extracts (63 64) Contaminating tumor cells in these filtrates seem to have been ruled out but the nature of the transfer mechanism—infection versus transduction—is in doubt The results of Gross with leukemia have been repeated by Woolley with specific genotypic combinations of donor and host (G W Woolley unpublished observations) Law *et al* (108) were able to obtain pleiomorphic tumors of parotid gland origin in certain test mice following the introduction of cell free leukemic material According to Gross "parotid virus" is supposedly present in leukemic as well as normal tissue extracts

The cell free transmissions of Gross were particularly successful in the C3H strain some lines of which share a histocompatibility factor with AKs the carriers of the presumed leukemia virus Recently Gross found that certain sublines of C3H are susceptible others are resistant to this "virus This remarkable genetic relationship with virus sensitivity led Buffett Sorenson and Furth (unpublished data) to investigate on the one hand the host range of the "virus" and on the other hand the host range of AK cells Their preliminary survey indicates that the agent in AK leukemia extract injected into newborn mice enhanced the leukemia incidence in the following strains C3Heb DBA/1 RF BALB/C and also AK all individuals of which are supposed to carry the agent Even on the assumption that cells were present in these extracts the results cannot be explained by tissue grafting for the induced leukemias violated anticipated host barriers in transplantation assays Most of them

did not behave as AK tumors but were transplantable to both donor and recipient strains They appeared to be "ambivalent"

Studies with lysogenic bacteria and bacteriophages serve as reminders that "virus infection is also a species of genetic recombination" (110) Leukemia transmission from a high leukemic to a low leukemic mouse strain might well be a kindred type of transduction wherein normally nonleukemogenic viruses function as vectors of endogenous "trans missible mutagens" postulated by Murphy (132, 133) for avian virus tumors

Many cases of accidental hepatitis choriomeningitis and other viral infections of transplantable tumors have been recorded (20 26 96 107 117 136 162) but no genetic modification of tumor cells as a conse quence of these virus infections was found Nevertheless it may be worth while to infect deliberately leukemic mouse stocks whose tissues lack the transmitting agent with viruses of limited cytopathogenicity and then to test for altered transmission potential In discussing viruses the heterogeneity and elusiveness of agents classified under this name should be kept in mind

Unequivocal evidence for the transport or exchange of genetic par ticles between mammalian cells will depend on the judicious use of metabolic and iso antigenic characteristics (102) Fortunately the latter are dominant The difficulty of detecting recessive traits incorporated into the diploid or polyploid chromosome sets of tumor cells limits ex perimental prospects Ascites tumor clones with genetically stable features which might prove useful as markers are available however Certain clones can be identified by cytoplasmic criteria chromosome types and numbers and specific biochemical or antigenic properties (Table II) The labeled cell lineages are viable in frozen storage at −76 (22) genetic drift *in vivo* prior to transduction tests can thus be minimized Phagocytosis in certain leukemias (114 143) provides a natural means of feeding nuclear fragments of one genotype to cells of another Immunologic incompatibility of the donor tissue in a host environment which matches the recipient cells would serve to neutralize cellular contamination of the chromatin fragments

Through the opportunistic combination of all these possibilities lymphosarcoma transmission with nuclear fractions and oncologic trans duction problems in general can now be attacked with genetic precision Forecasting the potentialities of somatic tissue genetics Lederberg (110) believes that only experiments of the type outlined are likely to resolve the genetic basis of differentiation and of neoplasia

## Closing Comments

Gorer (57) deplores that despite the generally acknowledged useful ness of transplantable tumors there has been a tendency to regard ex periments with tumor grafts as somewhat old fashioned and this field as more or less closed An abundance of new uses for these old tools is however apparent from our survey Even a skeptical appraisal of contemporary work in rodent leukemia should dispel utilitarian mis givings (83) transplanted mouse leukemia is still the "best experimental tool for studying the complex problems of the chemotherapy of leu kemia (25 89)

Among the new methods and concepts those borrowed from micro biology are especially apt to advance leukemia research In the recent past the transplantation field has graduated from the 16 gauge trocar to the micromanipulator from tissue mash to single cell clone from the ill defined "white laboratory mouse" to isogenic resistant lines from diameters of tumor lumps to variance analysis for somatic mutation Through improvements in cytologic and immunologic techniques the infant science of somatic tissue genetics has come into its own Knowl edge of the neoplastic leukocyte gained on the cellular level and on the more obscure level of host restraint cannot fail to reinforce clinical investigation

REFERENCES


1 Allen S L *Genetics* 40 627 (1955)
2 Ambrus J L Ambrus C M Back N Johnson G C and Harrisson J W E *Proc Am Assoc Cancer Research* 1 2 (1953)
3 Amos D B *Brit J Exptl Pathol* 34 464 (1953)
4 Amos D B *Brit J Cancer* 9 216 (1955)
5 Amos D B *Ann N Y Acad Sci* 63 706 (1956)
6 Amos D B Gorer P A and Mikulska Z B *Proc Roy Soc* B144 369 (1955)
7 Amos D B Gorer P A and Mikulska Z B *Brit J Cancer* 9 209 (1955)
8 Amos D B and Day E D *Ann N Y Acad Sci* in press
9 Andreasen E *Anat Record* 100 634 (1948)
10 Arvy L *Le Sang* 16 198 (1944)
11 Barrett M K *Cancer Research* 12 535 (1952)
12 Barrett M K and Deringer M K *J Natl Cancer Inst* 11 51 (1950)
13 Bernard J Mathey J and Leprat J *Semaine hôpitaux Paris* 28 3736 (1952)
14 Bierman H R Kelly K H Cordes F L Petrakis N L Foster M and Lose E A *Federation Proc* 10 281 (1951)
15 Biesele J J *Cancer Research* 7 70 (1947)
16 Biesele J J and Gasic G *Cancer Research* 7 65 (1947)
17 Billingham R E Brent L and Medawar P B *Nature* 172 603 (1953)
18 Borges P R F and Kvedar B J *Cancer Research* 12 19 (1952)

19 Borges I R F Kvedar B J and Foerster G E *J Natl Cancer Inst* **15** 341 (1954)
20 Braunsteiner H and Friend C *J Exptl Med* **100** 665 (1954)
21 Braunsteiner H and Pakesch F *Acta Haematol* **14** 181 (1955)
22 Breedis C and Furth J *Science* **88** 531 (1938)
23 Brncic D and Hoecker G *Bol soc biol Santiago Chile* **7** 88 (1950)
24 Brncic D Hoecker G and Gasic G *Acta Unio Intern contra Cancrum* **7** 761 (1952)
25 Burchenal J H *in* Leukaemia Research" Ciba Foundation Symposium pp 13 23 Little Brown and Co Boston 1954
26 de Bruyn W M *Cancer Research* **9** 395 (1949)
27 Dougherty T F *Physiol Revs* **32** 379 (1952)
28 Dunham L J and Stewart H L *J Natl Cancer Inst* **13** 1299 (1953)
29 Dunn T B *J Am Med Womens Assoc* **6** 411 (1951)
30 Dunn T B *J Natl Cancer Inst* **14** 1281 (1954)
31 Engelbreth Holm J Spontaneous and Experimental Leukemia in Animals (translated by C L Heel) Oliver and Boyd Edinburgh and London 1942
32 Felix M D and Dalton A J *J Natl Cancer Inst* **16** 415 (1955)
33 Finch S C Ross J F and Ebaugh F G Jr *J Lab Clin Med* **42** 555 (1953)
34 Fischer S B Penavlillo S Pizarro O and Hoecker G *Biologica* **15** 7 (1951)
35 Foley E J *Proc Soc Exptl Biol Med* **80** 671 (1952)
36 Foley E J *Proc Soc Exptl Biol Med* **80** 675 (1952)
37 Fouts P J *J Indiana State Med Assoc* **46** 971 (1953)
38 Furth J *Blood* **6** 964 (1951)
39 Furth J *Proc Inst Med Chicago* **19**(5) (1952)
40 Furth J *Cancer Research* **13** 477 (1953)
41 Furth J *in* Leukaemia Research Ciba Foundation Symposium pp 38 41 Little Brown and Co Boston 1954
42 Furth J and Strumia M *J Exptl Med* **53** 715 (1931)
43 Furth J Seibold H R and Rathbone R R *Am J Cancer* **19** 521 (1933)
44 Furth J and Kahn M C *Am J Cancer* **31** 276 (1937)
45 Gardner M V *J Franklin Inst* **243**(1) 77 (1947) **243**(2) 1 (1947)
46 Gardner W U Dougherty T F and Williams W L *Cancer Research* **4** 73 (1944)
47 Gasic G Hoecker G Pizarro O Maturana H and Mardones E *Proc 4th Intern Congr Hematol Mar del Plata* 185 (1953)
48 Gillam A G *Blood* **8** 693 (1953)
49 Goldie H *Ann N Y Acad Sci* **63** 711 (1956)
50 Goldie H and Felix M D *Cancer Research* **11** 73 (1951)
51 Goldie H Butler C H Anderson M M Maxwell M C and Hahn P F *Cancer Research* **13** 125 (1953)
52 Goldie H Jeffries B R Green S S and Simpson M R *Cancer Research* **14** 423 (1954)
53 Gordon A S *Ann N Y Acad Sci* **59** 907 (1955)
54 Gorer P A *Brit J Exptl Pathol* **17** 42 (1936)
55 Gorer P A *J Pathol Bacteriol* **44** 691 (1937)

56 Gorer P A *J Pathol Bacteriol* **54** 51 (1942)
57 Gorer P A *Brit J Cancer* **9** 103 (1948)
58 Gorer P A *Brit J Cancer* **4** 372 (1950)
59 Gorer P A *Ann N Y Acad Sci* **63** 882 (1956)
60 Gorer P A Lyman S and Snell G D *Proc Roy Soc* **B135** 499 (1948)
61 Gorer P A and Mikulska Z B *Cancer Research* **14** 651 (1954)
62 Gorer P A and Amos D B *Cancer Research* **16** 338 (1956)
63 Graffi A Bielka H Fey F and Heine U *Naturwissenschaften* **42** 421 (1955)
64 Gross L *Acta Haematol* **13** 13 (1955)
65 Hauschka T S *Cancer Research* **12** 615 (1952)
66 Hauschka T S *Proc 2nd Natl Cancer Congr Cincinnati* **2** 1044 (1952)
67 Hauschka T S *Trans N Y Acad Sci* [2] **16** 64 (1953)
68 Hauschka T S *J Natl Cancer Inst* **14** 723 (1953)
69 Hauschka T S *J Natl Cancer Inst* **15** 1530 (1955)
70 Hauschka T S *Transpl Bull* **2** 154 (1955)
71 Hauschka T S and Levan A *Exptl Cell Research* **4** 457 (1953)
72 Hauschka T S and Kvedar B J *Proc Am Assoc Cancer Research* **1** 19 (1954)
73 Hauschka T S and Schultz J *Transpl Bull* **1** 203 (1954)
74 Hauschka T S and Grinnell S T *Proc Am Assoc Cancer Research* **2** 22 (1955)
75 Hauschka T S Kvedar B J Grinnell S T and Amos D B *Ann N Y Acad Sci* **63** 683 (1956)
76 Hauschka T S Amos D B and Grinnell S T *Proc Am Assoc Cancer Research* **2** 116 (1956)
77 Hewitt D *Brit J Prevent & Soc Med* **9** 81 (1955)
78 Hoecker, G *Transpl Bull* **1** 203 (1954)
79 Hoecker G Counce S and Smith P *Proc Natl Acad Sci U S* **40** 1040 (1954)
80 Hoecker G Pizarro O Brncic D and Gasic G *Cancer Research* **15** 9 (1955)
81 Hoecker G and Hauschka T S *Transpl Bull* in press
82 Hogreffe G *Brit J Cancer* **2** 108 (1948)
83 Israels M C G in Leukaemia Research Ciba Foundation Symposium pp 26 32 Little Brown and Co Boston 1954
84 Kaliss N and Newton O *Anat Record* **105** 535 (1949)
85 Kaplan H S *Cancer Research* **14** 535 (1954)
86 Kidd J G and Todd J E *Proc Soc Exptl Biol Med* **86** 781 (1954)
87 Kieler J *Rev hematol* **10** 516 (1955)
88 Kirschbaum A *Cancer Research* **11** 741 (1951)
89 Kirschbaum A Geisse N C Judd T and Meyer L M *Cancer Research* **10** 762 (1950)
90 Klein G *Exptl Cell Research* **2** 291 (1951)
91 Klein G *Exptl Cell Research* **2** 518 (1951)
92 Klein G *Cancer Research* **12** 589 (1952)
93 Klein G *Ann N Y Acad Sci* **63** 741 (1956)
94 Klein G Klein E and Révesz L *Transpl Bull* **1** 102 (1954)
95 Koprowska I *Ann N Y Acad Sci* **63** 738 (1956)

96 Koprowski, H *Ann N Y Acad Sci* **54** 963 (1952)
97 Koprowski H *Nature* **175** 1087 (1955)
98 Koprowski H *Ann N Y Acad Sci* **63** 895 (1956)
99 Korteweg R Z *Krebsforsch* **29** 455 (1929)
100 Law L W *Cancer Research* **2** 108 (1942)
101 Law L W *Cancer Research* **4** 257 (1944)
102 Law L W *Cancer Research* **14** 695 (1954)
103 Law L W *J Natl Cancer Inst* **15** 817 (1954)
104 Law L W *in* "Leukaemia Research Ciba Foundation Symposium pp 105-126 Little Brown and Co Boston 1954
105 Law L W *Advances in Cancer Research* **2** 281 (1954)
106 Law L W and Boyle P J *Proc Soc Exptl Biol Med* **74** 599 (1950)
107 Law L W and Dunn T B *J Natl Cancer Inst* **11** 1037 (1951)
108 Law L W Dunn T B and Boyle P J *J Natl Cancer Inst* **16** 495 (1955)
109 Lederberg J *J Cellular Comp Physiol* **45** (Suppl.) 75 (1955)
110 Lederberg J *Ann N Y Acad Sci* **63** 662 (1956)
111 Lederberg J and Edwards P R *J Immunol* **71** 232 (1953)
112 Levan A and Hauschka T S *J Natl Cancer Inst* **14** 1 (1953)
113 Levan A and Hauschka T S *Hereditas* **39** 137 (1953)
114 Levine S *Science* **123** 185 (1956)
115 Lewis M R *Am J Cancer* **29** 510 (1937)
116 Lewis M R *Growth* **18** 97 (1954)
117 Lindorfer R K and Syverton T *Proc Am Assoc Cancer Research* **1** 33 (1953)
118 Little C C *in* Biology of the Laboratory Mouse (G D Snell ed) pp 279 309 Blakiston Co Philadelphia 1941
119 Lushbaugh C C and Steiner P E *Cancer Research* **9** 299 (1949)
120 Lynch C L *J Natl Cancer Inst* **15** 161 (1954)
121 McCarty M Taylor H E and Avery O T *Cold Spring Harbor Symposia Quant Biol* **11** 177 (1946)
122 MacDowell E C *Cold Spring Harbor Symposia Quant Biol* **11** 156 (1946)
123 MacDowell E C and Richter M N *J Cancer Research* **14** 434 (1930)
124 MacDowell E C Taylor M J and Broadfoot J *Carnegie Inst Wash Yearbook* **50** 200 (1951)
125 Mellors R C Hlinka J Kupfer A and Sugiura K *Cancer* **7** 779 (1954)
126 Menten M L Willms M and Wright W D *Cancer Research* **13** 729 (1953)
127 Miller R A and Taylor M J *Proc Soc Exptl Biol Med* **68** 336 (1948)
128 Mirand E A and Hoffman J G *Proc Soc Exptl Biol Med* **88** 656 (1955)
129 Mirand E A and Hauschka T S *Proc Am Assoc Cancer Research* **2** 134 (1956)
130 Mitchison N A *Nature* **171** 267 (1953)
131 Mitchison N A *Proc Roy Soc* **B142** 72 (1954)
132 Murphy J B *Trans Assoc Am Physicians* **46** 182 (1931)
133 Murphy J B *Bull Johns Hopkins Hosp* **56** 1 (1935)
134 Murphy J B and Sturm E *Cancer Research* **1** 379 (1941)
135 Nakic B and Nakic Z *Le Sang* **25** 844 (1954)
136 Nelson J B *J Exptl Med* **98** 433 (1953)

137 Nettleship A *Am J Pathol* 21 527 (1945)
138 Nungester W J and Fisher H *Cancer Research* 14 284 (1954)
139 Osgood E E Seaman A J Tivey H and Rigas D A *Rev hematol* 9 543 (1954)
140 Paschkis K E Cantarow A and Stasney J *J Natl Cancer Inst* 15 1525 (1955)
141 Pedersen E *Opera domo biol hered human Univ Hafniensis* (Copenhagen) 36 1 (1955)
142 Potter J S Taylor M J and MacDowell E C *Proc Soc Exptl Biol Med* 37 655 (1938)
143 Rebuck J W and Crowley J H *Ann N Y Acad Sci* 59 757 (1955)
144 Revesz L and Klein G *J Natl Cancer Inst* 15 253 (1954)
145 Richter K M *Ann N Y Acad Sci* 59 863 (1955)
146 Sachs L and Gallily R *J Natl Cancer Inst* 15 1267 (1955)
147 Sachs L and Gallily R *J Natl Cancer Inst* 16 803 (1956)
148 Schrek R and Preston F W *J Natl Cancer Inst* 16 1021 (1956)
149 Schweitzer M D and Furth J *Am J Cancer* 37 224 (1939)
150 Seligmann M Grabar P and Bernard J *Le Sang* 26 52 (1955)
151 Shelton E *J Natl Cancer Inst* 15 49 (1954)
152 Snell G D *J Natl Cancer Inst* 13 719 (1952)
153 Snell G D *J Natl Cancer Inst* 14 691 (1953)
154 Snell G D and Borges P R F *J Natl Cancer Inst* 14 481 (1953)
155 Snell G D Smith P and Gabrielson F *J Natl Cancer Inst* 14 457 (1953)
156 Stasney J Cantarow A and Paschkis K E *Cancer Research* 10 775 (1950)
157 Stasney J Cantarow A and Paschkis K E *Ann N Y Acad Sci* 54 775 (1952)
158 Stewart S E *J Natl Cancer Inst* 14 1187 (1954)
159 Stoerk H C *J Biol Chem* 171 437 (1947)
160 Stoerk H C and Emerson G A *Proc Soc Exptl Biol Med* 70 703 (1949)
161 Sturm E and Murphy J B *Cancer Research* 4 384 (1944)
162 Taylor M J and MacDowell E C *Cancer Research* 9 144 (1949)
163 Todd J E and Kidd J G *Proc Soc Exptl Biol* 86 865 (1954)
164 Toolan H W *J Natl Cancer Inst* 14 745 (1953)
165 Weiss P *in* Biological Specificity and Growth (E G Butler ed ) pp 195 206 Princeton University Press Princeton New Jersey 1955
166 Willis R A "Pathology of Tumours C V Mosby Co St Louis 1948

# 6

# Genetic and Nongenetic Factors Influencing the Induction of Mouse Leukemia[1]

ARTHUR KIRSCHBAUM

Baylor University College of Medicine and the University of Texas M D Anderson Hospital and Tumor Institute
Texas Medical Center Houston Texas

The incidence of leukemia is high in certain inbred strains of mice in others the disease occurs rarely Even when the incidence is high certain members of an inbred strain remain leukemia free Nongenetic influences are probably responsible for the nonappearance of leukemia in a minority of animals of genetically homogeneous high leukemia stocks In the C58 strain the incidence of leukemia in the progeny of the nonleukemics is as high as in the offspring of mice which develop the disease indicating that the leukemics and nonleukemics are genetically similar (19)

Leukemia may be induced in low leukemia stocks by the administration of leukemogenic agents—x rays (2) carcinogenic hydrocarbons (21) and estrogenic hormone (3) Inherited factors are important in determining susceptibility to leukemogens Certain inbred strains of mice are highly susceptible to the leukemogenic action of specific agents whereas others do not respond to the same agents (11) Strain C58 which exhibits a high incidence of spontaneous leukemia does not respond to the leukemogenic action of methylcholanthrene whereas the low leukemia Dba/2 strain is very susceptible (Kirschbaum and Liebelt unpublished observations) Synergistic effects may be obtained when animals are susceptible to the independent action of each of two leukemo

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gens, suggesting that leukemia inciting agents may have a common path way of action

Genes determining susceptibility to leukemogenic action exert their influence in the target lymphoid or myeloid tissue Strong A mice are susceptible to the induction of lung and mammary tumors by methyl cholanthrene (26) but absolutely refractory to the induction of leu kemia (Kirschbaum and Mixer 15 Kirschbaum and Liebelt unpub lished observations) On the other hand Dba/2 mice resist the induc tion of lung tumors by the same chemical carcinogen but are susceptible to the genesis of both leukemia and mammary neoplasms Were sys temic metabolism of the carcinogen the gene determined factor then a differential response of target tissues to a single agent within one type of host might not be expected

Gross reports that when the leukemia virus is transmitted to "low leukemia strains immediately after birth leukemogenesis results (5) Carcinogen x ray or hormone induced leukemia appears in "low leu kemia" strains *if treatment is begun even months postpartum* The virus of mouse mammary cancer cannot be demonstrated in breast tumors induced in mice which have been rendered virus free by foster nursing (Kirschbaum and Liebelt unpublished observations) If a leukemia virus is implicated in *spontaneous* leukemogenesis it does not follow that a similar agent is involved in *induced* leukemia

For leukemia induction the mouse thymus plays an important role Furth (1) originally demonstrated its significance in the genesis of spontaneous leukemia the incidence was remarkably reduced in AK "high leukemia" mice which had been thymectomized Kaplan (7) showed that thymectomy similarly reduces the incidence of x ray induced leukemia of C57BL mice and Law and Miller (17) obtained a reduced incidence of methylcholanthrene induced leukemia in Dba/2 mice if the thymus had been extirpated Susceptibility to the leukemogenic action of methylcholanthrene does not reside essentially in the thymus how ever By regulating the dose of carcinogen it was demonstrated that the thymus possesses a lower threshold of sensitivity (12) and that gonad ectomy enhances susceptibility of Dba/2 mice to the induction of leukemia whether or not the thymus is present (14)

*Law and Miller (17) have obtained evidence that the function of the* thymus may be to provide a suitable environment for the transformation of normal to leukemic lymphocytes Normal thymus of a high leukemia parent strain was grafted into thymectomized $F_1$ hybrids between the high and a low leukemia strain spontaneous leukemia developed in high incidence in these $F_1$ hybrids *if they possessed normal thymus*

grafts from the high leukemia parent stock Thymus grafts from the low leukemia parent strain *did not stimulate leukemogenesis*

After the development of leukemia in Law s experiments transplantation studies revealed that the leukemic cells in the thymectomized $F_1$ hybrids originated from the host rather than the cells of the thymic grafts The graft apparently provided the inciting environment in which *leukemic transformation of host lymphocytes occurred* Genetic factors may thus influence a tissue environment specific for leukemogenesis

In similar experiments on x ray induction of leukemia Kaplan *et al* (9) have found that leukemic lymphocytes may arise from the thymic graft per se X ray induced leukemic transformation may occur in thymic grafts made after irradiation of the host the leukemogenic effects of x rays being "secondary via a humoral factor (9)

*The development of leukemia is dependent on nongenetic* as well as on genetic factors Nongenetic influences may either *initiate* or *modify* leukemogenesis As indicated above genetic factors determine whether or not a host responds to such *initiating* agents as methylcholanthrene or estrogenic hormone Factors which *modify* the genesis and the growth of leukemic cells are considered below

*Steroid hormones* may considerably influence leukemogenesis *Estrogenic hormone is independently leukemogenic in certain strains of mice* (3) If combined with x rays synergistic action may be demonstrated (16) Androgenic hormone may nullify the leukemogenic action of estrogens (3) or depress the activity of x rays (4 8) or methylcholanthrene (13) in inducing leukemia Cortisone may delay the appearance of spontaneous (28) x ray induced (10) or methylcholanthrene induced (Kirschbaum and Liebelt unpublished observations) leukemia *These effects have been obtained under specifically defined situations* involving the doses of agents and strains of mice so that generalizations are not in order

In AKR mice the inhibiting effect of androgens on spontaneous leukemogenesis has been demonstrated (23) The incidence may be higher and the age of onset earlier in the females of certain high leukemia strains (24) This discrepancy in onset between the sexes is especially *apparent in $F_1$ hybrids between the high leukemia C58 and certain low* leukemia strains (Kirschbaum and Liebelt unpublished observations)

Aging modifies susceptibility to leukemogens Whether this is the result of involution of target tissue induced by hormonal factors or an intrinsic alteration dependent on other influences has not been shown C57BL mice are highly susceptible to the leukemogenic effects of x rays only if the mice are irradiated prior to 6 months of age (6) Dba/2

mice respond to the leukemogenic action of methylcholanthrene only if treated relatively early in life Mice resistant as a result of aging may have their susceptibility restored by gonadectomy (Kirschbaum and Liebelt unpublished observations)

*Life expectancy* may be correlated with latent period of leukemogenesis $F_1$ hybrid mice live longer than pure strain high leukemia parents and the age of appearance of spontaneous leukemia is delayed (11) CBA mice have a longer life expectancy than Dba/2s and the latent period of development of leukemia is correspondingly longer (22)

A *maternal resistance factor* of StoLi mice is transmitted to $F_1$ progeny of this strain and high leukemia C58 males (18) This factor is passed on either transplacentally or through the breast milk it delays the onset of spontaneous leukemia and reduces its incidence $F_1$ hybrids with the StoLi rather than the C58 mothers developing less leukemia later in life When pure C58 mice were foster nursed by StoLi females a similar effect was obtained (20) Not only does this maternal resistance factor affect the development of leukemia but life expectancy is increased

*Calorie restriction* may as in the case of spontaneous mammary cancer of mice (27) decrease leukemia incidence (25) In experiments designed to inhibit the development of leukemia the factor of inanition must always be controlled For example administration of cortisone may depress the body weight and unless the dose is carefully regulated or pair fed animals are used as controls delay in onset of leukemia cannot be attributed to any specific action of cortisone

To summarize genetic factors influence the development of spontaneous and induced mouse leukemia Leukemogenic agents include ionizing radiations chemical carcinogens and estrogenic hormone Certain influences (e g administered cortisone) may modify the growth of leukemic cells although initiation of the disease is not inhibited Other factors may actually inhibit leukemogenesis (caloric restriction administered androgen thymectomy)

## REFERENCES

1 Furth J and Boon M C in A A A S Research Conference on Cancer p 129 American Association Advancement of Science Washington D C 1945
2 Furth J and Furth O B *Am J Cancer* 28 54 (1936)
3 Gardner W U Dougherty T F and Williams W L *Cancer Research* 4 73 (1944)
4 Gardner W U *Proc Soc Exptl Biol Med* 75 434 (1950)
5 Gross L *Proc Soc Exptl Biol Med* 76 27 (1951)
6 Kaplan H S *J Natl Cancer Inst* 9 55 (1948)
7 Kaplan H S *J Natl Cancer Inst* 11 83 (1950)

8 Kaplan H S and Brown M B *Cancer Research* 11 262 (1951)
9 Kaplan H S Brown M B Hirsch B and Carnes W H *Proc Am Assoc Cancer Research* 2 27 (1955)
10 Kaplan H S Brown M B and Marder S N *Cancer Research* 11 262 (1951)
11 Kirschbaum A *Cancer Research* 11 741 (1951)
12 Kirschbaum A and Liebelt A Cancer Research 15 689 (1955)
13 Kirschbaum A and Liebelt A *Proc Am Assoc Cancer Research* 2 28 (1955)
14 Kirschbaum A Liebelt A and Falls N C *Cancer Research* 15 685 (1955)
15 Kirschbaum A and Mixer H W *J Lab Clin Med* 32 720 (1947)
16 Kirschbaum A Shapiro J R and Mixer H W *Proc Soc Exptl Biol Med* 72 632 (1949)
17 Law L W and Miller J H *J Natl Cancer Inst* 11 425 (1950)
18 MacDowell E C *Science* 81 2105 (1935)
19 MacDowell E C *Cancer Research* 5 65 (1945)
20 MacDowell E C *Cancer Research* 15 23 (1955)
21 Mider G B and Morton J J Am J Cancer 37 355 (1939)
22 Mixer H W and Kirschbaum A *Radiology* 50 476 (1949)
23 Murphy J B *Cancer Research* 4 622 (1944)
24 Richter M N and MacDowell E C *Physiol Revs* 15 509 (1935)
25 Saxton J A Boon M C and Furth J *Cancer Research* 4 401 (1944)
26 Shapiro J A Study of Genetic Susceptibility to Urethane Induced Pulmonary Tumors of Mice Thesis University of Minnesota 1950
27 Tannenbaum A and Silverstone H *Advances in Cancer Research* 1 451 (1953)
28 Woolley G W and Peters B A *Proc Soc Exptl Biol Med* 82 286 (1953)

# 7

# Viral Etiology of Mouse Leukemia[1]

LUDWIK GROSS
Cancer Research Unit Veterans Administration Hospital
Bronx New York

It has been demonstrated during the past several years in our laboratory that mouse leukemia is caused by a filterable thermolabile agent which is transmitted in certain families of mice from one generation to another directly through the embryos (6-11)

Filtered (Berkefeld N or Selas O2 or O3) extracts prepared from leukemic donors (that is from either Ak or C58 mice which developed leukemia "spontaneously") and inoculated into newborn mice (less than 16 hours old) of a susceptible but essentially nonleukemic strain (such as *C3H or C57 Brown) induced typical generalized leukemia after a* latency varying from several months to more than a year (Table I and Fig 1) (6-15)

When Ak leukemic *cell* suspensions were inoculated into newborn C3H mice leukemia resulted within 2 to 4 weeks (5) This leukemia however was apparently the result of multiplication of the implanted Ak cells leukemic cell suspensions prepared from such C3H donors could be transplanted to adult healthy mice of the Ak strain but not to adult mice of the C3H (recipient) line (Table II)

On the other hand when leukemia was induced in C3H mice by inoculation of cell free extracts (injecting newborn C3H mice with centrifuged or filtered Ak extracts and inducing leukemia after a latency of several months) the resulting leukemia could be transplanted to adult mice of the C3H (recipient) line but could not be grafted back to the *Ak donor strain (Table III)*

These transplantation experiments suggest that leukemia induced in

[1] This research was aided in part by a grant from the Damon Runyon Memorial Fund

C3H mice by inoculation of centrifuged or filtered Ak extracts consists of the recipient host s cells changed into leukemic cells by the pathogenic action of the inoculated cell free agent

Heating of the extracts to 68 for ½ hour inactivated their pathogenic potency (11 13)

TABLE I

INOCULATION OF FILTERED (SELAS OR BERKEFELD) AK LEUKEMIC EXTRACTS INTO NEWBORN C3H OR C3H(F) MICE OF TWO SUBSTRAINS

| | Fresh extracts | | | Heated extracts | | |
|---|---|---|---|---|---|---|
| Substrain | Number inoculated | Number developing leukemia† | Number developing parotid tumors† | Number inoculated | Number developing leukemia† | Number developing parotid tumors† |
| Bittner C3H | 138 | 32(23%) | 24 | 73 | 0 | 1 |
| Bittner C3H(f) | 182 | 58(32%) | 9 | 93 | 2 | 4 |
| Bittner total | 320 | 90(28%) | 33 | 166 | 2 | 5 |
| N C I C3H(f) | 162 | 7( 4%) | 9 | 45 | 0 | 0 |

One half hour at 65 to 68

† Average age leukemia 11 months parotid tumors 5 months
Average age of mice dying without tumors or leukemia 16 months

TABLE II

TRANSPLANTATION OF LEUKEMIC CELL SUSPENSIONS (FROM SPONTANEOUS TRANSPLANTED OR INDUCED LEUKEMIA) INTO ADULT MICE OF AK AND C3H STRAINS

| Leukemic donor | | Results of inoculation of adult recipient mice | |
|---|---|---|---|
| Donor s strain | Donor s leukemia resulted from | Strain Ak | Strain C3H |
| Ak | Spontaneous | Positive | Negative |
| C3H | Inoculation of Ak leukemic cells | Positive | Negative |
| C3H | Inoculation of cell free Ak agent† | Negative | Positive |

Ak leukemic cells grafted into newborn C3H mice cause leukemia in 2 to 3 weeks

† After inoculation of cell free Ak extracts into newborn C3H mice leukemia develops after 3 to 24 months

TABLE III

BASIC DIFFERENCES BETWEEN TRANSPLANTED (BY CELL GRAFT) AND INDUCED (BY INOCULATION OF CELL FREE EXTRACTS) LEUKEMIA

| Method | Results of inoculation into mice of: Same strain (Ak) | | Susceptible foreign strain (C3H or C57 Brown) | | Incubation time months | Leukemic tumor at site of inoculation | Resulting leukemia can be transplanted to |
|---|---|---|---|---|---|---|---|
| | Newborn | Adult | Newborn | Adult | | | |
| Transplantation of leukemic cells | Positive | Positive | Positive | Negative | ½–1 | Usually present | Donor strain |
| Inoculation of cell free Ak leukemic agent | ?† | Negative | Positive | Negative | 3–21 | Absent | Recipient strain |

Centrifuged 7000 × g or filtered

† Possibly accelerated development of "spontaneous" leukemia

After centrifugation at 144 000 × g for periods of time varying in a recent series of experiments from 15 minutes to 4½ hours the pathogenic potency of the extracts was with only few exceptions limited to the sediment pellet (22)



FIG 1 This C57 Brown female (No 150 Expt 2507 A) developed typical generalized leukemia at the age of 14 months as a result of inoculation when less than 3 hours old with supernatant of a centrifuged (7000 × g) Ak leukemic extract Note very large leukemic notes under chin in axillary and inguinal pits also very large spleen

The leukemic tissues could be preserved by dry freezing or stored in 50% glycerin for at least several months retaining their pathogenic potency When resuspended centrifuged and inoculated into newborn C3H mice such extracts induced leukemia parotid gland tumors or soft tissue sarcomas (18 20)

The leukemic agent could be passed by cell free inoculations of newborn hosts for 6 consecutive passages thus far After each of the

first 3 passages the latency period appeared to be shortened suggesting increased potency of the recovered extract (20)

*These experiments suggest that leukemia in mice is caused by a filterable thermolabile agent which has the ability to multiply and to induce specific symptoms of disease these characteristics are those of a pathogenic virus*

Extracts prepared from normal embryos of healthy animals of leukemic lines (such as Ak or C58) were found to contain the leukemic agent inoculation of such extracts into newborn susceptible mice resulted in the development of leukemia It is apparent therefore that the leukemic agent is transmitted from one generation to another directly through the embryos (8 16 20)

The fact that the filtered or centrifuged leukemic extracts inoculated into newborn mice of a susceptible strain induced leukemia in some animals but parotid gland tumors or soft tissue sarcomas in others (Figs 2 and 3) suggests two possibilities either one single agent might be able to induce leukemia or other types of malignant tumors or the extract used for inoculation contained a mixture of several distinct oncogenic agents (13 20)

Only on very rare occasions did mice develop both leukemia and parotid tumors In most instances those among the inoculated mice that developed leukemia had neither parotid tumors nor sarcomas On the other hand parotid tumors developed occasionally in the same animals in which simultaneously or shortly afterward subcutaneous sarcomas also appeared

Spontaneous leukemia is extremely rare in untreated mice of our C3H strain the incidence is below 0 5% Parotid tumors and subcutaneous sarcomas are virtually unknown in untreated C3H mice of the subline used in our laboratory

These experiments have been repeated at the National Cancer Institute (23) and more recently also at the Sloan Kettering Institute (25) When filtered Ak or C58 leukemic extracts were inoculated by Law and his co workers into newborn C3H mice of the National Cancer Institute subline parotid tumors or subcutaneous sarcomas could be induced but very few leukemias appeared among the inoculated mice The National Cancer Institute subline of the C3H strain appears therefore to be substantially less susceptible to the leukemogenic action of the extracts than the Bittner subline of the same C3H inbred strain used in our laboratory (17) Accordingly when Woolley recently repeated the same experiments at the Sloan Kettering Institute in New York, using newborn C3H mice of the susceptible Bittner substrain for inoculation

of the filtered extracts either leukemia parotid tumors or subcutaneous sarcomas could be induced (25)

The fact that the leukemic extracts inoculated into newborn C3H mice were able to induce either leukemia parotid tumors or soft tissue sar



Fig 2. Bilateral parotid gland tumors developing in a 12 month old *C3H(f)* female (No 18 Expt 2275-C) as a result of inoculation when less than 15 hours old with filtered (Berkefeld) Ak leukemic extract

comas is puzzling If we assume that a single agent may be able to in duce such different types of neoplasms this fact alone would be of fundamental importance On the other hand if we assume that the injected extracts contain a mixture of several distinct oncogenic agents such an assumption also may be of basic importance an association of

several distinct oncogenic agents may not be coincidental the possibility of a synergistic action might be considered

*In recent experiments it was observed that when the leukemic extracts were mixed in vitro with ethyl ether the ability of such extracts to induce leukemia was with rare exceptions destroyed (21)* On the other hand the potency of the ether treated extracts to induce either



FIG 3 Bilateral parotid tumors developing in a C3H female (No 110 Expt 2709 B) at the age of 4 months as a result of inoculation when less than 15 hours old with supernatant from centrifuged (7000 × g) Ak leukemic extract diluted 1 100 000 Note typical appearance of these tumors similar to clusters of grapes on both sides of the neck

parotid tumors or sarcomas did not appear to be appreciably impaired Should the leukemic extracts used for inoculation contain three distinct oncogenic agents it would be possible to speculate that the ether treatment in vitro destroyed the more sensitive leukemic agent without apparently affecting the more resistant parotid tumor and sarcoma agents

There were basic differences among the three different types of neo

plasm induced with the leukemic extracts The leukemias induced with the cell free extracts were typical generalized in most instances lymphatic usually involving the blood and bone marrow and transplantable in practically 100% into adult mice of the recipient (C3H) strain

The parotid tumors occasionally developed very early and were usually bilateral composed of grapelike clusters of tumors arising in



FIG 4 Photomicrograph of a parotid gland tumor which developed in a C3H(f) male (No 363 Expt 2709 C) at the age of 4½ months as a result of inoculation when less than 15 hours old with supernatant from centrifuged (7000 × g) Ak leukemic extract diluted 1 100 (Hematoxylin and eosin × 95)

multiple foci in the parotid glands (Fig 3) In initial phases of development these tumors were of adenocarcinomatous type (Fig 4) but later bands of cells resembling sarcomas could be found between the acinar clusters In more advanced phases of development the carcinomatous character of these tumors was for all practical purposes lost the tumors assuming purely sarcomatous structure The parotid tumors could not always be transplanted to adult C3H mice even when successfully

transplanted for one two or several successive generations subsequent transfers would frequently grow poorly or not at all

Finally the subcutaneous sarcomas developing usually in older animals were hard white infiltrating tumors very difficult to dissect and grind Microscopically there were fibrosarcomas (Figs 5 and 6) fibromyxosarcomas giant cell tumors etc When transplanted into adult C3H mice they grew very poorly taking only occasionally and after a considerable delay of several weeks or months A few however could

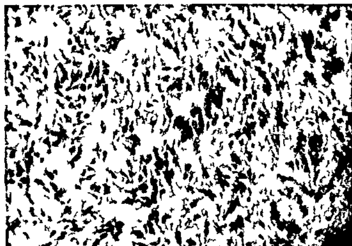


Fig 5 Primary fibrosarcoma This hard infiltrating subcutaneous tumor developed on the back of a C3H male (No 404 Expt 2806-E) at 10 months of age This mouse was inoculated with filtered (Selas 03) extract prepared from parotid gland tumor The parotid gland tumor used for the preparation of the extract had been induced with Ak leukemic extract (Hematoxylin and eosin × 200)

be transplanted successfully retaining their initial microscopic appearance

With rare exceptions the induced C3H leukemia could not be transplanted to adult mice of the Ak strain (11) None of the parotid tumors or sarcomas could be transplanted into adult Ak mice

In an attempt to recover the agent the C3H mice in which leukemia was induced by inoculation of cell free Ak leukemic extracts were used as donors of leukemic tissues serving for the preparation of leukemic extracts Such centrifuged or filtered C3H leukemic extracts were then inoculated into newborn C3H mice As a result some of the inoculated mice developed leukemia and others developed parotid tumors or subcutaneous sarcomas (18) (Table IV)

In other series of experiments C3H mice in which parotid tumors were induced by inoculation of cell free Ak leukemic extracts were used as donors Parotid tumors removed from these mice were ground with physiological saline solution centrifuged (7000 × g) and in some in stances also filtered These extracts were then inoculated into newborn C3H mice As a result some of the inoculated mice developed typical generalized leukemia and others developed parotid tumors and/or sub cutaneous sarcomas (18) (Table V)

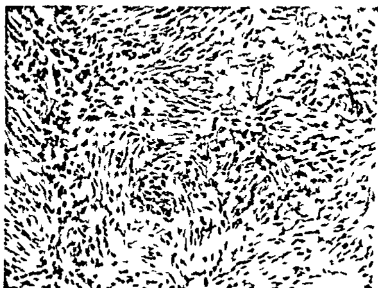


FIG 6 Fibrosarcoma which developed subcutaneously in the right axillary pit in a C3H(f) male (No 363 Expt 2709 C) at 4 months of age This mouse was inoculated when less than 15 hours old with centrifuged (7000 × g) Ak leukemic extract diluted 1 100 This mouse developed also a parotid tumor (Hematoxylin and eosin × 200)

Finally cell free centrifuged or filtered extracts were prepared from the induced subcutaneous sarcomas These extracts were then inocu lated into newborn C3H mice These experiments are still in progress Among the inoculated mice however some have already developed either parotid tumors or subcutaneous sarcomas In other instances typical generalized leukemia has developed (22) in the inoculated mice

It appears therefore that leukemia in mice is caused by a sub microscopic agent presumably a virus which is transmitted directly through the germinal cells from one generation to another In most in stances this agent would be so well adapted to its carrier host that it would cause no symptoms of disease (16)

TABLE IV

INOCULATION OF CELL FREE EXTRACTS FROM INDUCED C3H LEUKEMIAS INTO NEWBORN C3H AND C3H(F) MICE

| Number of extracts | Strain | Number of mice inoculated† | Number developing leukemia‡ | Number developing parotid tumors‡ | Number developing sarcomas† |
|---|---|---|---|---|---|
| | C3H | 84 | 21 | 9 | 3 |
| | C3H(f) | 40 | 20 | 3 | 0 |
| 18 | | 124 | 41‡ | 12§ | 3 |

Part of the data in this table have been published previously (18) however these experiments have been continued and additional mice have developed leukemia parotid tumors and/or sarcomas The adjusted data reflect results available at this time

† Average age leukemia 7 months parotid tumor 5 months sarcomas 11 *months*

‡ Two developed parotid tumors also

§ Four developed subcutaneous sarcomas also

TABLE V

INOCULATION OF CELL FREE EXTRACTS FROM INDUCED PAROTID TUMORS INTO NEWBORN C3H OR C3H(F) MICE

| Number of extracts | Number of mice inoculated | Number developing leukemia† | Number developing parotid tumors† | Number developing sarcomas† |
|---|---|---|---|---|
| 38 | 300 | 49 | 23‡ | 28 |

* Part of the data in this table have been published previously (18) however these experiments have been continued and additional mice have developed leukemia parotid tumors and/or sarcomas The adjusted data reflect results available at this time

† Average age leukemias 14 months parotid tumors 6 months sarcomas 15 months

‡ Of these 6 developed parotid tumors and subcutaneous sarcomas 2 developed parotid submax and adrenal tumors

It is quite possible to assume that under normal conditions of life the host parasite relationship would be so well balanced that the change of the agent from a symbiont to that of a pathogenic parasite would occur only very occasionally perhaps once in many generations of the carrier hosts Thus the incidence of leukemia developing spontaneously in descendants of the same family and caused by a direct transmission

of the same virus might be separated by few or many generations of perfectly healthy hosts even though such animals may be carrying and transmitting the seeds of disease

By selective inbreeding of either descendants or close relatives of leukemic mice it was possible eventually to develop families carrying a leukemic agent of high pathogenic potential Gradually by repeating such a selection over a period of years it was possible to obtain lines such as the Ak (2 4) and C58 (24) strains of mice not only carrying the agent but actually developing active disease in practically every generation

For the preparation of extracts if a leukemic donor is selected from a family in which leukemia develops only exceptionally the leukemic virus present in such extracts will probably be of low pathogenic potency When injected into susceptible hosts it may induce leukemia only on rare occasions This is an example of a stable symbiont type of leukemic agent with a tendency to exist on perfect terms with its carrier host comparable perhaps to a lysogenic phage

On the other hand an extract prepared from a leukemic donor of a family such as Ak or C58 in which leukemia develops frequently and at early age will usually contain a leukemic agent of high pathogenic potential If inoculated under proper experimental conditions into a susceptible host such an agent may induce a high incidence of leukemia

It is apparent that cell free transmission of mouse leukemia can be accomplished in the laboratory only under certain rather limited experimental conditions Only newborn mice can be used for inoculations preferably less than 12 hours old and if possible less than 6 hours old When animals more than 16 hours old are used for inoculations the results become erratic and the development of leukemia or tumors considerably delayed if it occurs at all (This appears to be true at least for this form of leukemia which occurs in Ak and C58 strains of mice )

Furthermore the animals must be of a susceptible strain The genetic susceptibility is obviously of fundamental importance since differences between sublines of the same inbred line may be sufficient to determine success or failure of the experiment (17) Furthermore some strains such as the C3H appear to be susceptible to the induction of either leukemia parotid tumors or subcutaneous sarcomas whereas mice of the C57 Brown inbred line inoculated under apparently identical experimental conditions develop leukemia (15) but only on rarest occasions parotid gland tumors (20)

The leukemic agent appears to be rather labile and extracts must be

inoculated promptly after preparation in any event within 48 hours having been kept in the meantime at 0

The ability of a cell free extract to induce leukemia appears to depend on the amount of infective virus This may vary to a considerable degree according to the donor from which the tissues are collected for the

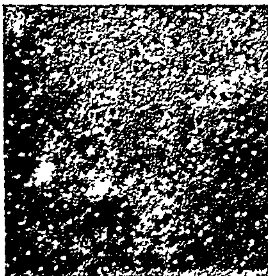


FIG " Electron mi rograph of a leukemic extract prepare l from a C3H mouse in whicl leukemia was induced with a potent extract (The potency of the extract was increased by several successive cell free passages through newborn hosts ) The extract for electron microscopy was prepared from leukemic spleen liver and lym phoid tumors After preliminary centrifugation this extract was filtered first through Selas candle then through Gradocol membrane porosity 740 mμ and then through membrane 140 mμ Drop preparation fixed with osmic acid chromium shadowed (RCA Electron Microscope EMU mag × 24 750) Numerous spherical particles varying in diam ter from 30 to 70 mμ (This electron micrograph was prepared with the assistance of Mr Marvin D Boatright )

preparation of such an extract In this respect mouse leukemia would not be different from chicken lymphomatosis (3) or the Rous sarcoma (1) The richer the extract in infective virus the higher the incidence of induced leukemia and the shorter the latency period

If an extract relatively high in infective virus is to be prepared the leukemic donors should be selected among those Ak or C58 mice which developed spontaneous leukemia early in life rather than among older ones

More potent extracts however able to induce leukemia after a relatively shorter latency period could be prepared from leukemic organs of C3H or C57 Brown mice in which leukemia had been induced by inoculation of cell free Ak or C58 leukemic extracts Recently it was found that the potency of the leukemic extracts could be considerably increased by passing the agent through several successive generations by means of cell free inoculations into newborn hosts (20)

Recent experiments still in progress in which Gradocol membranes have been used for the filtration of the leukemic extracts suggest that the mouse leukemia agent is a particle less than 100 mμ in diameter (22) These preliminary results appear to be consistent with our current experiments dealing with the sedimentation of the leukemic agent in an ultracentrifuge (22) Electron microscopic studies carried out in our laboratory appear to indicate that the leukemic extracts contain particles having a diameter somewhere between 30 and 70 mμ (Fig 7) Whether these particles actually represent the leukemic agent(s) remains yet to be determined

In summary mouse leukemia was found to be caused by a filterable thermolabile agent transmitted in certain families of mice from one generation to another directly through the embryos In this respect mouse leukemia would be essentially similar to chicken lymphomatosis (3)

The fact that filtered extracts prepared from mouse leukemia and inoculated into newborn mice of certain susceptible strains may induce either leukemia parotid gland tumors or subcutaneous sarcomas requires further study The leukemic agent may be able to induce different types of neoplasms On the other hand the leukemic extracts may contain a mixture of several distinct oncogenic agents

REFERENCES

1 Bryan W R *J Natl Cancer Inst* **16** 285 (1955)
2 Cole R K and Furth J *Cancer Research* **1** 957 (1941)
3 Cottral G E Burmester B R and Waters N F *Poultry Sci* **33** 1174 (1954)
4 Furth J Ferris H W and Reznikoff P *J Am Med Assoc* **105** 1824 (1935)
5 Gross L *Cancer* **3** 1073 (1950)
6 Gross L *Proc Soc Exptl Biol Med* **76** 27 (1951)
7 Gross L *Proc Soc Exptl Biol Med* **78** 342 (1951)
8 Gross L *Ann N Y Acad Sci* **54** 1184 (1952)
9 Gross L *Cancer* **5** 620 (1952)
10 Gross L *Acta Haematol* **10** 18 (1953)
11 Gross L *Cancer* **6** 153 (1953)

12 Gross L *Cancer* **6** 948 (1953)
13 Gross L *Proc Soc Exptl Biol Med* **83** 414 (1953)
14 Gross L *Blood* **9** 557 (1954)
15 Gross L *Proc Soc Exptl Biol Med* **86** 734 (1954)
16 Gross L *Acta Haematol* **13** 13 (1955)
17 Gross L *Proc Soc Exptl Biol Med* **88** 64 (1955)
18 Gross L *Proc Soc Exptl Biol Med* **88** 362 (1955)
19 Gross L *Rev hématol* **10** 509 (1955)
20 Gross L *Cancer* **9** 778 (1956)
21 Gross L *Acta Haematol* **15** 273 (1956)
22 Gross L experiments to be published
23 Law L W Dunn T B and Boyle P J *J Natl Cancer Inst* **16** 495 (1955)
24 MacDowell E C and Richter M N *Arch Pathol* **20** 709 (1935)
25 Woolley C W and Small M C *Cancer* **9** 1102 (1956)

# Added Comment

L W LAW
National Cancer Institute Bethesda Maryland

The previous discussions relating to etiologic factors in leukemogenesis point up the lack of any definitive unifying concept There are many etiologic agents related in some manner to the induction of lymphomas in experimental animals radiations carcinogenic hydrocarbons estrogens possibly adrenal and pituitary hormones and possibly viral like agents

It is recognized that for any given morphologic form of neoplasm—that is skin carcinomas lung adenomas hepatomas and so on—many different carcinogenic agents are capable of their induction Are we to assume that the underlying neoplastic response is the same in all cases? This has never been proved although in the leukemias certain of the leukemogenic agents are known to act synergistically at below threshold levels (6)

Too little attention has been given to the different morphologic forms of lymphomas *in the experimental animal In the mouse the common* form is the lymphocytic neoplasm but in addition there are found granulocytic leukemia plasma cell neoplasms types A and B neoplasms (monocytic histiocytic and Hodgkin s like lesions) mast cell neoplasms and so forth

Should these be considered *biologically* as well as clinically and morphologically independent diseases? Is such a classification one of convenience only or is it related to basic principles of etiology? In the DBA/2 strain of mice for example fractionated irradiation induces preponderantly lymphocytic neoplasms of thymic origin whereas carcinogenic hydrocarbons induce the whole gamut of morphologic forms of lymphomas (*Potter unpublished observations*)

One would inquire for example in Dr Gross s work, whether he is talking about the induction of lymphocytic neoplasms or other forms There exists at the present time a subline of C3H mice which does have a fairly high incidence of lymphomas—40 to 50%—so called subline C3H/

Fg These are preponderantly nonlymphocytic (2 8) To date there is no evidence of a viral etiology in this subline (8)

To re emphasize one point that appeared in the literature years ago unmistakable evidence shows that susceptibility to leukemia in the mouse has a genetic basis (11) The early work of MacDowell using crosses between a high leukemic C58 and a low leukemic STOLI strain is of particular interest

The problem in any genetic analysis in which hereditability is not Mendelian is to determine whether the animals in a generation in which reassortment of genes is occurring are uniform or diverse in their potentialities to produce leukemia If one assumes the presence within a high leukemic strain of mice—the AKR strain for example—of a nongenic cellular pathogen this would in all likelihood be maintained at a uniform level in the strain by inbreeding On crossing with a low leukemic strain it would be reduced to lower levels

Thus it would be expected that all individuals in a second hybrid generation—for example a backcross—would have uniform potentialities of developing leukemia If genes were responsible for the differences between high and low leukemic strains the mice in a second hybrid generation would be a genetically diverse group since there occurs reassortment of genes in crossbreeding depending on the distribution of chromosomes

The "progeny test" was used by MacDowell in classifying backcross mice (high × low leukemic strains) as to their genetic uniformity or diversity The classification was based on the incidence of leukemia in families of mice obtained by mating backcross males to low leukemic females (and not on a plus or minus description of individual mice) Significant differences in the incidence of leukemia among families were found ranging from 0 to 43% If more than one gene is involved in susceptibility a continuous variation (rather than bimodal) is expected and indeed this has been found

This information suggests the existence of many different genotypes among the backcross males tested and provides a genetic basis for susceptibility Additional evidence is found in linkage tests Susceptibility to leukemia has been found in at least two linkage groups of the mouse (7 10)

Such evidence does not rule out the possibility of nongenetic variables playing a decisive role in the etiology of leukemia however The mammary tumor milk agent in mice plays a major role as an etiologic agent in certain experimental situations yet in other situations it appears non effective As shown by Heston *et al* (4) this viruslike agent is under

strict genetic control as far as its propagation and transmission are concerned On the other hand 40% mammary carcinomas have been observed in mice of a particular $C3H_f$ subline in which the milk agent is known not to be present as far as can be determined by biologic tests (5)

Of three known major etiologic factors concerned with the induction of mammary carcinoma in mice a combination of any two will realize the potentialities of carcinoma No single factor however has been shown to cause in all or none difference under any set of conditions In delineating the etiologic factors in leukemia one has to keep these experimental observations in mind

Thus it remains to be determined what the major known factors are in the etiology of leukemia Genetic susceptibility or perhaps resistance —and there is some evidence that resistance to leukemia has a genetic basis (that is resistance to the spontaneous occurrence of leukemia)— must play a significant role

Any discussion of the etiology and pathogenesis of lymphocytic neoplasms in the mouse must consider the role of the thymus Removal of this organ nearly completely overrides the potentialities of developing lymphocytic neoplasms in the spontaneous (AK AKR C58) carcinogen induced (DBA) and radiation induced (C57BL B × A) disease *The part thymic tissue plays in other morphologic forms of* lymphoma in mice remains to be determined Nonetheless this tissue appears to be a *sine qua non* for the development of lymphocytic neoplasms since transfer of thymic tissue to the subcutaneous connective tissues expresses the potentiality of the mouse to develop the neoplasm Paradoxically in many cases the transferred thymic tissue does not supply the progenitor cells of the neoplasm but has the characteristics of a genetically determined "sphere of influence from which the neoplasm arises (9)

Dr Gross has implicated a viruslike agent as one of the etiologic factors in lymphocytic neoplasms in mice Its role in relation to genetic susceptibility to carcinogens and to irradiation and so on should be considered Does irradiation for example inactivate a latent virus? I know of no evidence in this regard for any neoplasm

A more critical evaluation should be made of the observations reported from the laboratories of Graffi and colleague (1 3) who report the induction in mice of leukemia (particularly in one report 30% chloromas) after injection of filtrates of Ehrlich carcinoma *Sarcoma 37 and* Landschutz mouse Sarcomas I and II There also exists a report in the literature of a probable relationship between LCM virus and lym

phomas (13) This was a study with non inbred Princeton RI mice and the differences between control and experimental groups were not large

Finally to re-emphasize what Dr Hauschka stated a suggestion of the operation of a transformation phenomenon (speaking in terms of bacterial transformation) has been offered by Stasney and colleagues (12) where the tumorogenic quality itself is allegedly transferred by DNA preparations (at least chromatin preparations) The validity of these observations is predicated on the purity of the chromatin fraction (purity re absence of cells)

The present evidence appears to be against any such interpretation as far as the observations of Stasney and colleagues are concerned but the possibility of transformations relating to leukemia should be critically pursued The availability of ascitic neoplasms and the analogy between these and microorganisms especially *E coli* needs hardly be stressed There appears much to be gained in pursuing the analogy both in method and in concept

One of the difficulties however in pursuing such an analogy has been the lack of suitable markers in somatic cells It is now possible to use in certain ascitic lymphomas markers such as histocompatibility markers and resistance to antileukemic drugs Work in this particular field will be rather rewarding

## REFERENCES


1 Bielka H Graffi A and Fey F *Naturwissenschaften* **42** 399 (1955)
2 Figge F *Proc Am Assoc Cancer Research* **1** 13 (1954)
3 Graffi A and Schmidt F *Deut Gesundheitsw* **9** 1309 (1954)
4 Heston W E Deringer M K and Andervont H B *J Natl Cancer Inst* **6** 289 (1945)
5 Heston W S Deringer M K Dunn T B and Levillain W D *J Natl Cancer Inst* **10** 1139 (1950)
6 Kirschbaum A *Cancer Research* **11** 741 (1951)
7 Law L W *J Natl Cancer Inst* **12** 1119 (1952)
8 Law L W and Dunn T unpublished observations
9 Law L W and Potter M *Proc Natl Acad Sci U S* **42** 160 (1956)
10 MacDowell E C Potter J S and Taylor M J *Cancer Research* **6** 65 (1945)
11 MacDowell E C and Richter M N *Arch Pathol* **20** 709 (1935)
12 Stasney J Cantarow A and Paschkis K E *Ann N Y Acad Sci* **54** 775 (1952)
13 Traub E *Z Bacteriol* **147** 16 (1941)

# General Discussion

DR EDWIN E OSGOOD (Portland Oregon) As Dr Law *pointed out there is* need for a unifying concept of the leukemias lymphomas and cancer Since 1950 we have had such a concept which was very briefly presented in the *Annals of the New York Academy of Sciences* [59 806 (1955)] on leukocytic functions and it is now being prepared for detailed publication

*If we make the assumption that the chemically* mature *differentiating cell* produces an inhibitor of cell division then any change in the cell that leads to a shortened life span of this differentiating cell would lead to failure of production of this in hibitor This shortening of the life span may be caused by alterations in the genetic material of the cell in turn produced by ionizing radiation heredity viruses or viruslike bodies or carcinogens There seems to be incontrovertible evidence that each of these factors is a predisposing cause in some and not in other cases of cancer

We have shown [*Acta Haematol* 13 145 (1955)] that the life span of the cell is shorter in chronic granulocytic leukemia than in the normal cell It is common knowledge that you do not see the mature cell in the more acute of these tumors and you can get a change to a still shorter life span

Among the Metazoa one cell resulting from division has to go on to differentiate and die while the other remains immature to divide again The inhibitor described would act as an automatic regulator of cell number

Much evidence exists for such an inhibitor of mitosis If you scrape off a tiny bit of skin you haven t added anything—you have subtracted something but the cells underneath begin to divide more rapidly If you give blood to the Red Cross you haven t added anything but the cells in the marrow begin to divide more rapidly If you take out two-thirds of the liver in a parabiotic rat you haven t added but even the liver of the parabiont begins to show multiplication We have analogs of such short range growth regulators in the inducers and organizers of embryology

I think we should begin to look at the mature normal cell for an inhibitor of cell division and there may be inherent in the immature cell capable of division the initial potentialities of the unicellular organism that does undergo uncontrolled ex ponential growth if the environment is suitable

This hypothesis if correct would show that all the current concepts of cancer etiology are correct but that each of the factors—heredity ionizing radiation chemi cal or physical carcinogens—acts as one of the predisposing causes to a genetic change leading to loss of an enzyme system necessary for a normal life span of the differ entiating cell and thus to failure of production of the specific inhibitor of logarithmic cell production produced by that differentiating cell

DR CHARLOTTE FRIEND (New York New York) In line with Dr Gross s work are some recent findings in our laboratory We have isolated a filterable agent from a Swiss mouse it is transmissible to adult mice of the same strain and induces a leu kemia Dr Furth has been kind enough to look at some of the histological sections and he says it appears to be of granulocytic type This is different from Dr Gross s which appears to be of a lymphatic type

DR GEORGE W WOOLLEY (New York New York) Some interest has been ex pressed in our attempts to repeat the work of Dr Gross and *I might say that through*

the kindness of Dr Charlotte Friend we have had some experience with her agent also These studies investigating the relation of cell free extracts prepared from leukemic tissues—centrifugates and filtrates—to the induction of cancer particularly leukemia in mice were started because of the interesting observations made by Gross that cell free viruslike material prepared from either leukemic tissue or normal tissue from leukemic strain mice would when given to newborn mice of different susceptible strains induce leukemia and other forms of cancer *In the meantime* a new and different cell free agent has been discovered by Dr Charlotte Friend of Sloan Kettering Institute

In general the development of leukemia or *other types* of tumors appeared to depend to a large extent on the preparation used and the genetics of the host receiving the preparation

In the first study of thirty one extracts (twenty of Akn Gs origin and eleven of C3H$_f$/Gs origin) a total of thirteen (six Akn and seven C3H) have been active in inducing leukemias parotid gland tumors and sarcomas The most active preparations were extracted from filtrate induced tumors rather than from spontaneous tumors One such preparation (filtrate) has induced 4 leukemias out of 5 inoculated C3H$_f$/Bi mice (80%) and 10 out of 17 C3H$_f$/Gs mice (59%)

Use of a supernatant which was diluted with an amount estimated to be 2 1 (two parts of supernatant to one *part of saline*) after 7,000 × g centrifugation was followed by only parotid gland tumors and sarcomas—6 and 8 respectively out of 17 injected C3H$_f$/Gs and 1 sarcoma out of 3 injected C3H/Gs mice

Use of one Akn centrifugate was followed by 5 leukemias out of 12 injected C3H$_f$/Gs mice *Another* Akn preparation was refrigerated at 4 in an ice water bath for 79 hours before use Of the 4 *C3H$_f$/Bi* mice injected with this extract 4 parotid gland tumors and 3 sarcomas resulted No tumors *have* occurred in the 6 C3H$_f$/Gs *mice* which were injected with this extract within 48 hours after preparation

In the entire study no leukemias have occurred in the same individual in which parotid gland tumors *and* sarcomas have occurred

Two leukemias in C3H$_f$/Gs mice were *the* only tumors to develop in the 293 control mice other than breast tumors in the unfostered (milk *factor*-carrying) mice C3H/Gs and C3H/Wy No leukemias were observed in 143 breeding C3H mice of various sublines at a mean age of 11 months nor were any leukemias observed in a group of 53 C3H mice now at a mean age of 13 months which were used as control animals in another experiment

All types of tumors were transplantable Tumors arising in C3H mice were transplantable to other C3H mice of the proper subline but not to Akn mice the strain of extract origin C3H$_f$/Gs tumors were transplantable to C3H$_f$/Bi but not to C3H$_f$/He mice

Out of a *total of* 245 C3H mice of the Bittner substrains treated with extracts 45 (18 4%) developed leukemia *10* (*4 1%* developed parotid gland tumors and 10 (4 1%) developed sarcomas an over all total of 65 (26 5%) malignant tumors Of 84 *C3H mice* of other sublines 1 (1 2%) developed leukemia and none developed sarcomas or parotid tumors Of the 188 control C3H mice of the Bittner substrains 2 (1 1%) developed leukemia but no leukemia *developed* in the 65 control mice of the other C3H substrains These experiments are being continued

Dr Friend called my attention to the difficulty of transferring the cell free leu

kemic agent under study by her to a foreign strain such as strain C3H It was transmissible adult to adult in the Swiss strain its strain of origin a point we have confirmed By means of inoculation into the newborn we have been able to transfer the agent and disease to strain C3H Here as in the Swiss mouse it resembles granulocytic leukemia as shown by positive peroxidase reaction of the cell granules After transfer to strain C3H it was transmissible adult to adult in this new host and also back to the adult Swiss mouse It is evident that this latter agent differs greatly from the agent or agents discovered by Dr Gross

It seems to me that a fascinating area for work has been opened up

Dr Arthur Kirschbaum (Houston Texas) We have had experiments under way attempting to confirm the work of Dr Gross We obtained from Dr Bittner the proper C3H subline and have injected newborn mice with extracts according to the methods outlined by Dr Gross Our series has run just about as long as Dr Woolley s Up to this time we have found no induced leukemias Dr Woolley tells me that the Bittner subline which he uses and in which he obtains positive results does not have the milk agent Ours does This of course could be a complicating factor

Dr George W Woolley In the material presented 31 mice had the milk factor Of those 31 none so far has exhibited leukemia Many of these are of the subline C3H/Wy the susceptibility of which is unknown as far as the leukemia factors are concerned

Chairman Bierman Dr Law would you like to comment a bit about the C3H resistant strain? That seems to be a potent complication here

Dr L W Law (Bethesda Maryland) I am not sure it is a complication There are probably fifteen or twenty different sublines of C3H and one would expect them to be different genetically I think it all depends on the sample one takes Dr Dunn in the National Cancer Institute has found an incidence of as high as 10% lymphomas in one of the sublines of the National Cancer Institute I believe if one keeps the animals long enough and pays as much attention to the controls as to the experimental groups one probably will get between 5 and 10%

Since this other work has come up I might say that we also have used some of Bittner s subline without the agent These animals are now 9 or 10 months along and we didn t start this until Dr Gross a year or so ago found that there was a subline difference in susceptibility We have found so far three early leukemias at an average age of 6 months in one litter Curiously enough the mother of this litter also had leukemia at 12 months of age These are the only positive results we have at the present time

I would like to ask a question of Dr Friend as to whether these so-called granulocytic leukemias grow progressively in Swiss mice Certain strains of mice certainly have a tendency to show extramedullary myelopoiesis Do these grow progressively in Swiss mice?

Dr Roger Klein (Galveston Texas) I have a question to ask Dr Gross Why do you think that your so-called virus is an exogenous agent and not an endogenous factor? Graffi in his last paper in *Klinische Wochenschrift* writes that the agent of *mouse* leukemia seems bound to the small mitochondria but he does not conclude

between the endogenous or exogenous origin. Perhaps there is in a normal cell two kinds of cytoplasmic particulates and, as Dr. Osgood said, a factor of inhibition and a factor of proliferation, and cancer could be an escape from the mechanism of homeostasis by the loss of the factor of inhibition and the multiplication of the other factor.

Dr. Ludwik Gross (Bronx, New York): When investigating an obscure disease it *is very difficult to judge whether we are dealing with a virus. If we can transmit* the disease by inoculating a *filtered* extract prepared from diseased tissues; if such an extract will lose its potency by heating; if the activity of the extract can be sedimented by ultracentrifugation; if the same disease can be reproduced in a *susceptible animal by inoculating such an extract, and then if again from the induced* disease another extract can be prepared which, in turn, will reproduce the same disease in another host, then we may be justified to assume that we are dealing with a transmissible, filterable virus.

The mouse leukemia agent is filterable, thermolabile, can be sedimented in an ultracentrifuge, and can be passed from one host to another. It apparently is a virus. The fact that the same filtered leukemic extract inoculated into newborn mice can induce leukemia or parotid tumors and/or soft tissue sarcomas could be explained by assuming that the extract contained a single agent able to cause different neoplasms. I am inclined to feel, however, that we are dealing with a mixture of three or more related but individually distinct oncogenic agents. The presence of several oncogenic agents in the leukemic host may not be purely coincidental. A synergistic action may be involved. Should it be possible to separate such agents and inoculate them individually, it may then be difficult, if at all possible, to induce leukemia. There are so many unknowns at this time that it is difficult to decide what we are dealing with until more facts become available.

Dr. Charlotte Friend: In answer to Dr. Law's question on myeloid metaplasia, this agent is transmissible, and myeloid metaplasia has never been proved to be transmissible; *also our virus results in a fatal disease.*

# 8

# Environmental Factors in Human Leukemia

L J WITTS
Nuffield Department of Medicine University of Oxford
Oxford England

There are only three agents or agencies which have been shown to affect the incidence of human leukemia They are heredity benzol and ionizing radiations and the evidence for heredity and benzol is not yet such as to convince a sceptic In what follows therefore I shall have to say rather more about plans for collecting evidence than about established facts

The revival of interest in research on leukemia in man is due partly to the introduction of new therapeutic agents which can arrest the course of the disease It is also due however to the apparent rise in incidence of leukemia and the evidence that cases are occurring as a result of exposure to ionizing radiations Before taking up these points however I ought to say something about classification

We cannot assume that the etiological factors will be the same for all types of leukemia The difference in the age and sex incidence of lymphatic and myeloid leukemias is so striking that it seems almost certain that they differ in causation Lymphatic leukemia is a disease of the extremes of life but more particularly of old age It is also a disease of males Myeloid leukemia is a disease of the middle span of life and it affects males and females equally The percentage of myeloid forms in leukemia occurring after irradiation is much higher than in spontaneous leukemia in man Some people believe that acute and chronic leukemia are different diseases and certainly their response to treatment is very different It is therefore certain that we need precise classification in studies of hereditary and environmental factors in leukemia Hematologists will usually base their diagnoses on the definitions given by the Committee for Clarification of the Nomenclature of Cells and

Diseases of Blood Forming Organs (4) but these are too detailed for many statistical purposes

The two parameters which must always be used in classifying leukemias as each of them is important are the acuteness of the disease process and the type of cell affected The International List of Causes of Death had hitherto concentrated too exclusively on cell type At the Seventh Revision of the International List which took place in 1955 there was a rearrangement of the heading 204 which deals with leukemia and the subdivisions are now as follows

204 0–Lymphatic leukemia
204 1–Myeloid leukemia
204 2–Monocytic leukemia
204 3–Acute leukemia (*this will include acute leukemia unqualified* and any condition in 204 0 and 204 1 if specified as acute but acute monocytic leukemia will stay in 204 2)
204 4–Other and unspecified leukemias

The effect of this is that in 204 0 and 204 1 one should place only the chronic forms of lymphatic and myeloid leukemias and in 204 3 all the acute leukemias irrespective of reputed cell type with the exception of monocytic leukemia This classification is therefore still not quite so detailed as some of us would like As far as the United Kingdom is concerned the General Register Office is able to give us the number of acute myeloid (myeloblastic) and lymphoid (lymphoblastic) leukemias as further breakdowns of 204 3 Although there are difficulties in identifying the type of cell in acute leukemia most hematologists believe that the correct diagnosis can be made in 80% of cases

Let us turn now to the figures for the incidence of leukemia The rate of increase of leukemia as a recorded cause of death has been greater than that of any other disease except lung cancer and coronary thrombosis The last figures published by the Metropolitan Life Insurance Company (11) show a rise from 1 8 to 4 7 per 100 000 in their policy holders between 1930 and 1951 My colleague David Hewitt last year published an analysis of the incidence of deaths attributed to leukemia in England and Wales (10) and he found that in 1953 the incidence was more than two and one half times as high as in 1931 As between different countries the incidence follows the order United States and Denmark > England Wales and Scotland > Western Germany and Ireland As between different regions of England it is higher in the South than in the North the South of England has for many years been more prosperous than the North As between economic *strata* it is higher in the rich than the poor As between races it is higher in the

white than the nonwhite In all five of these contrasts the sexes have been affected to an approximately equal extent, and the oldest age group has been affected more than any other The increase is in lymphatic leukemia It is particularly important to emphasize this because the etiological factors we recognize—benzol and ionizing radiations—produce myeloid leukemia

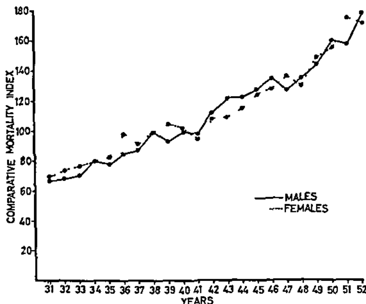



FIG 1 Leukemia Course of comparative mortality index for males and females in England and Wales from 1931 through 1952 (1938 = 100) Data collected by Hewitt (10)

The question at once arises whether these changes reflect merely the more frequent detection of the condition In other words would not many of these patients have died under a different diagnosis in the past? I can well remember one of the patients I had as a student who died with the diagnosis of oral sepsis and who was probably suffering from monocytic leukemia This interpretation is supported by the fact that both in the United States and in Great Britain the recorded death rate is highest in those groups of the population which presumably have access to the best medical facilities Because there is this doubt studies of the local variations of incidence within the United Kingdom or attempts to correlate them with surveys of the radioactivity of the soil and water are probably premature On the other hand it is unfortunate

that we do not know more about the geographical pathology of the disease in the broader sense Reliable figures for the incidence of the different types of leukemia at different ages in the tropics and subtropics would be of much interest In Japan and in the indigenous population of North and South Africa there appears to be a notable deficiency of chronic lymphatic leukemia which is not explained by the different age

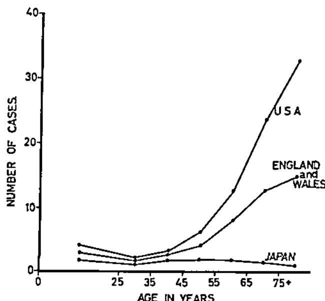



FIG 2 Deaths from leukemia per 100 000 in successive age groups in the United States England and Wales and Japan in 1952

structure of the populations Figure 2 charts the deaths from leukemia per 100 000 of the population taken from the Annual Epidemiological and Vital Statistics of the World Health Organisation (18) The disparity is in the later age groups and in chronic lymphatic leukemia

We need not waste time discussing whether the increase in leukemia in Western Europe and North America is apparent or real because that is something time itself will show The improvement in the health services in Great Britain has been so widespread and uniform that it is becoming increasingly difficult for anyone to die with symptoms of anemia without having an expert blood count Let us assume for a moment that there has been a real increase Men and women have been equally affected so that the cause is unlikely to be smoking cosmetics or occupation Cohort analysis—that is to say analysis of people according to the year in which they were born—indicates that the environ

mental factors responsible for this increase must have been operating for at least twenty five years This suggests that we cannot inculpate the sulfonamides or the antibiotics If the social class difference is a real one possible causes might be different dietary habits and greater ex posure to diagnostic x rays

Any analysis of possible environmental factors in leukemia is clearly going to meet formidable difficulties even if we limit it to factors which have come into action since 1870 I have always been impressed by Custer s (7) demonstration of the role of atabrine in the production of aplastic anemia Such an effect would never have been revealed with out a war to provide the appropriate populations of large bodies of troops with complete medical and statistical services In group I i e troops not taking atabrine the incidence of aplastic anemia was 0 04 and 0 18 per 100 000 troops per 6 month period In group II troops taking atabrine the incidence showed a precipitous rise from 0 66 to 2 84 cases per 100 000 per half year I say precipitous but let us think for a moment of the 99 997 not affected

Aplastic anemia is related to leukemia and occasionally precedes it (Bernard and Boiron la Dreyfus and Bessis 8) Etiological factors in leukemia may therefore be of the same degree of effectiveness as ata brine in aplastic anemia Even with the most massive irradiation com patible with survival the incidence of leukemia in man does not seem to be higher than 1 in 100 It is therefore not surprising that the scrutiny of hospital records of cases of leukemia such as Revol *et al* (15) carried out at Lyons has not revealed any precise etiological factors We have felt that the retrospective study of large numbers of case notes is not likely to be rewarding To discover anything of significance it seems necessary to have a planned investigation of a large group of cases with appropriately matched controls

The curious distribution of deaths from leukemia in childhood seems to offer a promising salient for attack on the etiological factors All reliable statistics of the incidence of leukemia show a moderate eleva tion in the first two years of life then an abrupt rise with the highe t incidence in the third and fourth years The curve falls steeply in the next three years and there is a slower progressive fall throughout the latter half of childhood Cooke (5) who was one of the first to draw attention to this phenomenon thought that the curve suggested that infection played a part in the initiation of leukemia It is noteworthy that most of the 1500 cases which he assembled must have occurred be fore the introduction of the sulfonamide drugs and therefore were not attributable to chemotherapy The life history of the child is short and

less confusing than that of the adult and if there are recognizable environmental factors in leukemia they may appear more clearly in children where they have not been overlaid by the other events and accidents of life It is just possible that we might light on etiological factors comparable with German measles during the mother s pregnancy

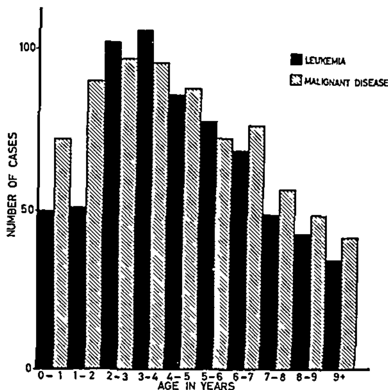



FIG 3 Ages at death of all children in England and Wales dying from leukemia and malignant disease in 1953 1954 and through June 1955

in children with congenital ocular defects or prematurity and oxygen therapy in retrolental fibroplasia

The curve of the age incidence of leukemia in childhood is not so peculiar to leukemia as is sometimes believed other forms of neoplastic disease in childhood have a similar age incidence In England it was felt that in a study of environmental factors in leukemia in childhood the children with leukemia should be compared not only with normal children but also with children with other forms of malignant disease We have concentrated our attention on the age group under 10 My

colleague Dr Alice Stewart, and her co workers in the Social Medicine Unit at Oxford have received from the General Registry Office the names and addresses of all children under the age of 10 dying from leukemia and malignant disease during the years 1953 1954 and up to June 1955 These amount to 670 cases of leukemia and 743 cases of malignant disease The age distributions are charted in Fig 3 and are seen to be closely alike

Dr Stewart is making arrangements to collect an equal number of controls matched for age sex and locality The parents of all the children dying of leukemia and malignant disease and all the 1413 controls will be interviewed and a printed questionnaire will be completed The main subjects of inquiry are the locality birth order previous medical history exposure to radiation food habits and home activities of the child Similar details will be obtained about the parents and a family history will be taken A brief summary of the fatal illness will also be made including treatment remissions and duration of survival A pilot study has already shown that it is possible to identify the cases and controls and to interview the parents without distress or difficulty This survey by Dr Stewart and her co workers should produce information of unique value on hereditary and environmental factors in the etiology of acute leukemia and on the natural history of the disease

The data on inheritance will be particularly valuable as opinions are at present so diverse Many examples of the familial occurrence of leukemia have been described and Anderson (1) has reported a remarkable family in which 5 out of the 8 children developed leukemia between the ages of 5 and 8 A few cases have also been described in twins (Cooke 6) It is the exceptional cases however which are likely to be reported and Morganti and Cresseri (13) as a result of a recent study say that any hereditary factor is negligible On the other hand Videbaek (16) who analyzed the family histories of 209 patients with leukemia and a corresponding number of controls concluded that the chance of a relative of a patient with leukemia getting leukemia was 1 in 250 as compared with 1 in 4000 of the control population What was inherited was a tendency to leukemia in general and not to a particular type of leukemia

In addition the incidence of malignant disease as a whole was higher in the relatives of patients with leukemia than in those of the controls Videbaek's work has been much criticized largely because it goes against the grain of the experience of most of us Of 81 hematologists consulted by Guasch (9) 53 had had no example of familial leukemia among their own patients The total number of cases of leukemia was

8586 of which 19 or 1 in 452 were consanguineous This is the sort of question which is bound to be decided sooner or later as the statistics of world health progressively improve

Our project for the study of etiological factors in the leukemia of childhood is still in its initial stage and it may well be that no con vincing information will be forthcoming We are on more certain ground when we turn to the evidence for the leukemogenic effect of ionizing irradiations The measurement of rads and roentgens of integral doses and megagram roentgens is a job for the physicist and I shall mention only a few figures for those who are as inexpert as I The total normal background radiation over a generation in man amounts to about 3 roentgens (Burch and Spiers 3) Workers with atomic energy are per mitted an aggregate exposure of 15 to 30 roentgens a year The ordi nary chest film exposes the patient to only a fraction of a roentgen and even with tomography the total exposure is small if simple filters are used On the other hand it has been calculated that a gastrointestinal examination exposes the patient to 15 roentgens a minute

Whatever the risk to the patient there can be no doubt about the risk to the operator Even though a gown and gloves are worn the upper part of the thorax and the head and neck are unprotected This is prob ably the reason why at all ages the risk of dying from leukemia is three times the normal in physicians Leukemia is eight to nine times as com mon in radiologists as in other physicians and dermatologists who use x rays and radium in treatment have an increased mortality The high mortality from leukemia in the profession as a whole is not explained by the particular occupational risk of radiologists and dermatologists but suggests that all doctors in their training and work come into contact with harmful amounts of ionizing radiations The minimum time for the development of the disease in medical practitioners is less than five years (Peller and Pick 14)

These figures for the incidence of leukemia in the medical profession have been obtained by somewhat imperfect statistical techniques The figures for the incidence of leukemia in the survivors of the atomic bombs which were dropped on Hiroshima and Nagasaki in August 1945 are better documented Up to the end of 1954 92 cases of leu kemia had been recognized (Moloney 12) In Hiroshima where it was possible to relate the development of leukemia to the distance from the hypocenter of the explosion and the severity of the radiation complaints the incidence of leukemia was 1 in 172 in the severely irradiated 1 in 3223 in those within 2500 meters of the hypocenter who were lightly irradiated or not irradiated and 1 in 12 912 in those who were more than

2500 meters from the hypocenter The peak incidence of leukemia was in 1950 1951 and 1952 and there was a sharp decline in 1953 Of the 92 cases 39 were chronic myeloid leukemia and only 1 was chronic lymphatic leukemia The remainder were acute and subacute leukemias with the myelogenous form again predominating though not to the same extent These of course are interim data and it would be premature to conclude that the full effect of the irradiation has yet been seen or that cases of chronic lymphatic leukemia will not appear at a later date

The third piece of evidence of the leukemogenic effect of ionizing radiations is the incidence of leukemia in patients treated by radiotherapy Most of the patients so treated have malignant disease and do not live long or else the dosage is small An exception is ankylosing spondylitis where a dose of 2000 roentgens measured on the skin is given to each of four fields (Windeyer 17) Ankylosing spondylitis is usually called rheumatoid arthritis of the spine in the United States though in fact it shows some clinical and serological differences from rheumatoid arthritis Brown and Abbatt (2) have collected information from all the radiotherapy centers in England Wales and Scotland and have analyzed the records of 9364 patients with ankylosing spondylitis who were treated by radiotherapy between 1940 and 1954 At the time of their publication they had obtained details of 25 patients who had developed leukemia Acute leukemia was much commoner than chronic and the myeloid was the most common cytological type a distribution closely comparable with what was found at Nagasaki and Hiroshima The incubation period was also similar being most often about five to seven years The observed deaths from leukemia were at least five times and possibly as many as ten times the expected number of such deaths The discrepancy was unlikely to be explained by an unusual susceptibility of patients with ankylosing spondylitis to the development of leukemia The incidence of leukemia was twice as common in those having two or more courses of treatment as in those having one which suggests that the radiation is the deciding factor This survey is continuing It has been expanded and made more complete It is not yet finished and the results are confidential but it is likely to demonstrate with statistical certainty the connection between leukemia and exposure to ionizing radiations

In summary then it has been proved that ionizing radiations can produce leukemia in man Nevertheless they do not seem to produce chronic lymphatic leukemia which is the main cause of the increased incidence of leukemia in recent years and in the more socially advanced

areas We must suspend judgment as to whether leukemia is actually increasing though it is quite certain that it is being diagnosed and recognized more frequently If we are to find anything about hereditary and environmental factors in human leukemia we must be prepared to work with larger populations and with higher standards of diagnosis than have been available to clinicians and epidemiologists up to the present time

REFERENCES

1 Anderson R C *Am J Diseases Children* **81** 313 (1951)
1a Bernard J and Boiron M *Le Sang* **25** 797 (1954)
2 Brown W M C and Abbatt J D *Lancet* **1** 1283 (1955)
3 Burch P R J and Spiers F W *Science* **120** 719 (1954)
4 Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood Forming Organs *Am J Clin Pathol* **20** 571 (1950)
5 Cooke J V *J Am Med Assoc* **119** 546 (1942)
6 Cooke J V *J Am Med Assoc* **152** 1028 (1953)
7 Custer R P *Am J Med Sci* **212** 211 (1946)
8 Dreyfus B and Bessis M *Le Sang* **25** 787 (1954)
9 Guasch J *Le Sang* **25** 384 (1954)
10 Hewitt D *Brit J Prevent & Soc Med* **9** 81 (1955)
11 Metropolitan Life Insurance Co *M L I Stat Bull* **36** 3 (1955)
12 Moloney W C *New Engl J Med* **253** 88 (1955)
13 Morganti G and Cresseri A *Le Sang* **25** 421 (1954)
14 Peller S and Pick P *Am J Med Sci* **224** 154 (1952)
15 Revol L Millet C and Thivollet A *Le Sang* **25** 825 (1954)
16 Videbaek A "Heredity in Human Leukaemia and Its Relation to Cancer" Arnold Busck Copenhagen 1947
17 Windeyer R B in Textbook of the Rheumatic Diseases (W S C Copeman ed ) 1st ed p 467 Livingstone Edinburgh 1948
18 World Health Organization Annual Epidemiological and Vital Statistics 1952 Geneva 1955

Part III

# Radiation Biology of Leukocytes

Chairman
Howard R Bierman
City of Hope Medical Center
Duarte California

# 9

# Radiation Injury and Regeneration in Lymphoid Tissues[1]

HENRY S KAPLAN and MARY B BROWN
*Department of Radiology Stanford University School of Medicine San Francisco California*

The thymus is important in the genesis of lymphosarcoma and lymphatic leukemia in the mouse (7 13) It has previously been found that such tumors are induced in significantly greater yield in strain C57BL mice when total body irradiation is given in multiple fractional doses spaced several days apart than when given daily or as a single dose (9) The experiments reported herein were undertaken to investigate the possibility that the pattern of injury and/or repair of the thymus after periodic irradiation differs in some characteristic way from that after daily fractionated treatment or single exposure Parallel observations were made on the superficial lymph nodes and spleen

Several groups have studied the sequence of events in acute radiation injury of the thymus and spleen particularly after single whole body x ray exposures (1 3 8 14) Under suitably controlled conditions decrease in thymic or splenic weight may be used as a biological dosimeter for the estimation of the comparative effectiveness of dissimilar types of radiations (5 6) Regeneration of irradiated lymphoid tissues has received less attention however (4) and there is little published information on their response to fractionated and intermittent doses of x irradiation (16)

## Experimental Procedure

Strain C57BL mice of both sexes aged 33 ± 3 days were carefully randomized and allotted to the appropriate number of groups in each experiment In our initial experiment the numbers of males and females

[1] These studies were supported by grants in aid from the National Cancer Institute the U S Public Health Service and the Jane Coffin Childs Memorial Fund for Medical Research

per group varied Thymic weight tends to be somewhat greater in females than males of this strain however (12) In later experiments therefore equal numbers of males and females were used Groups of 4 to 6 mice were designated for sacrifice at each of a series of time intervals during and after irradiation Total body x ray exposure was given to batches of 4 to 8 mice in a shallow perforated Lucite cylinder Physical factors of irradiation were 120 kvp 9 ma 0 25 mm Cu + 1 0 mm Al added filter 30 cm mouse target distance 32 r /min In experiment

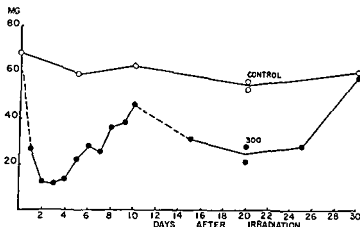



FIG 1 Mean wet weight of the thymus gland after 300 r of total body irradiation to C57BL mice

1 a single total body dose of 300 r was delivered In experiment 2 total body irradiation was given to three series of animals according to the following schedules series A received a single dose of 476 r series B four doses of 119 r each on successive days and series C four doses of 119 r each at 4 day intervals Additional randomized animals from the same litters were maintained untreated as age controls

At the predetermined time intervals groups of animals were sacrificed with ether weighed, and the thymus spleen and pooled superficial (usually two axillary and two inguinal) lymph nodes were excised carefully dissected free of connective tissue and fat weighed in the fresh state on a torsion balance and fixed in Bouin s fluid The tissues of two males and two females per group were sectioned and stained with hematoxylin and eosin for histologic study In the groups sacrificed 70 days or more after irradiation lymphoid tumors were encountered in all such instances more complete autopsies were performed and

other viscera in addition to the lymphoid tissues were preserved for microscopic examination

**Results**

Mean wet weights of the thymus spleen and pooled superficial lymph nodes after a single dose of 300 r are presented graphically in Figs 1 to 3

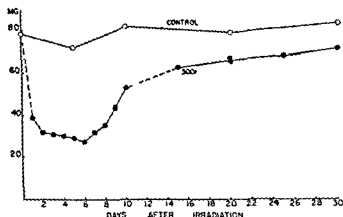



FIG 2 Mean wet weight of the spleen after 300 r of total body irradiation to C57BL mice

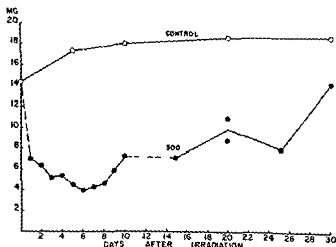



FIG 3 Mean wet weight of the superficial lymph nodes (two axillary two inguinal) after 300 r of total body irradiation to C57BL mice

Thymic weight falls precipitously reaching its lowest point 2 to 3 days after exposure at this dose level In contrast spleen and lymph node weights fall rapidly on the first day and more slowly thereafter passing through a minimum on days 5 to 6 Recovery of thymic weight also differs from that for spleen and lymph nodes The thymus increases in weight rapidly and steadily through the tenth day only to suffer a second involutional response between the tenth and fifteenth day persisting to day 20 with a renewed climb toward normal levels thereafter Spleen weight recovers steadily without a secondary phase of depletion and is essentially normal by day 20 Lymph node recovery is indolent and sluggish requiring about 30 to 40 days to return to normal levels after 300 r

In the fractional dose study (experiment 2) a generally similar thymic weight response was observed in both male and female mice as may be seen in Figs 4 and 5 The points have been plotted on a staggered basis such that the last day of irradiation coincides for all three series Initial injury was perhaps slightly greater after a single dose of 476 r than with daily or intermittent fractionated treatment There was again a distinct secondary drop in thymic weight most pronounced in series B and C beginning 8 to 10 days after the last x ray dose Sustained recovery resumed again at about day 25 and was complete in terms of thymic weight about 40 to 50 days after irradiation in all series High values in series C at later intervals are due to the more frequent occurrence of tumors in these mice as described below

Splenic weight fell somewhat more sluggishly than thymic weight with the minimum occurring 1 to 2 days later There is rapid regeneration after the eighth day with a dramatic rise to approximately twice normal levels at about 14 days after treatment Spleen weight then tends to settle back to the control level and remain there without the secondary drop which characterizes thymic weight response to irradiation There was no remarkable difference in the splenic weight curves of the three treated series Lymph node weight also decreases slightly more slowly than that of thymus and then recovers quite slowly with little tendency to overshoot" the control level during regeneration The lymph node weight curves of the three series were not considered to differ significantly

An interesting statistical phenomenon was noted during the repair phase Lymphoid tissue weights tended to be tightly clustered with small standard errors during the phase of injury Almost immediately thereafter some weights began to bounce upward again signaling the onset of regeneration but other animals from the same groups appeared

moribund and exhibited lymphoid tissue weights even lower than those noted during the phase of injury The group arithmetic means in such instances were associated with such large standard errors as to be meaningless *It appeared that these groups had segregated into two (or more) populations* one capable of survival and hematopoietic regeneration the other(s) incapable of recovery and apparently on the verge of death at the time of sacrifice

*Body weight exhibited no close relationship to the lymphoid tissue* weight responses except in the qualitative sense that it decreased slightly to moderately during the first 5 to 8 days after irradiation and then weight gain resumed The moribund animals however had all lost an extreme amount of weight and were obviously emaciated

*The initial histological appearance of the irradiated thymus* spleen and lymph nodes was generally consistent with previously published descriptions (14) Within 24 hours after irradiation most of the thymocytes exhibited pyknosis Cellular debris was abundant and macrophages were numerous *Damage was more severe in the cortex than in* the medulla resulting in the well known reversal appearance (Fig 6A and Figs 7A B) By 2 days after irradiation the cellular debris had been almost entirely removed from the cortex by macrophages (Fig 7C) *On the third or fourth day depending on dose repopulation of* the cortex by large immature lymphoid cells began quite abruptly apparently by proliferation and subsequent differentiation from large radioresistant nonlymphoid subcapsular cells (Fig 6B and Fig 7D E) *In the next several days the large lymphocytes were replaced ap*parently through a stepwise process of differentiation by progressively smaller more mature lymphocytes (Fig 7E–H) During this period mitotic figures were initially abundant and then became gradually sparse *By day 15 to 20 cortical and medullary architecture had been* restored almost to normal though the thymic lobes were still small (Fig 6D) Thereafter the only evident change consisted in an increase in over all dimensions of the lobes

*During this period two abnormalities were often noted* The boundary between the cortex and medulla was often poorly defined and in some instances the lymphocytes did not go through the sequence of differentiation and maturation cited above Instead much or all of the cortex *remained populated with cells which had a greater diameter* more abundant rather basophilic cytoplasm and a somewhat paler nucleus in which the chromatin was less densely matted and more vesicular than that of the normal thymocyte nucleus (Fig 8A) The resemblance *between these immature lymphoid cells and those of early lymphoid*

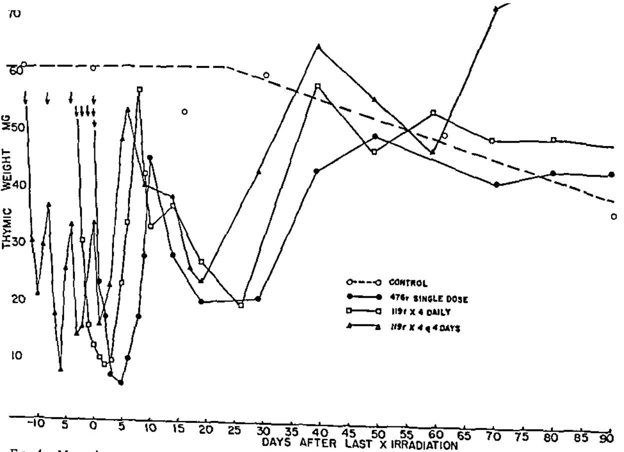



FIG 4 Mean thymic wet weight response of female C57BL mice during phases of injury and regeneration after 476 r of total body irradiation given as a single dose or in four equal fractions either daily or intermittently every 4 days Anomalously low weights encountered during the regenerative period in obviously moribund mice have been omitted

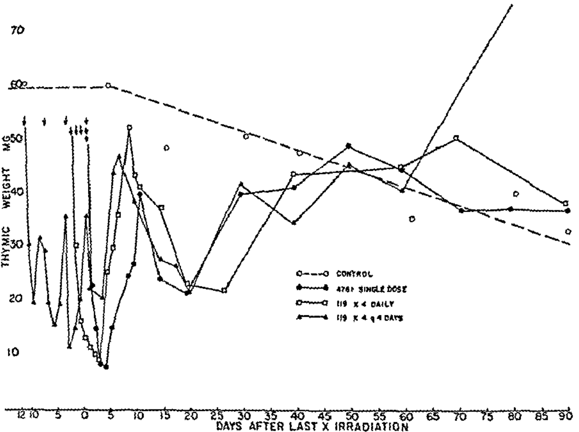



Fig 5 Same as Fig 4 for male C57BL mice Involution begins 3 to 4 weeks earlier in the normal males of this strain than in the females The responses are otherwise similar

t mors was highly suggestive particularly since mitotic figures remained plentiful in these instances A more detailed study of this apparent im pairment of differentiation and of its relation to the neoplastic trans formation is being made in collaboration with Dr William Carnes (2)

Thymic lymphoid tumors were encountered in some group sacrificed

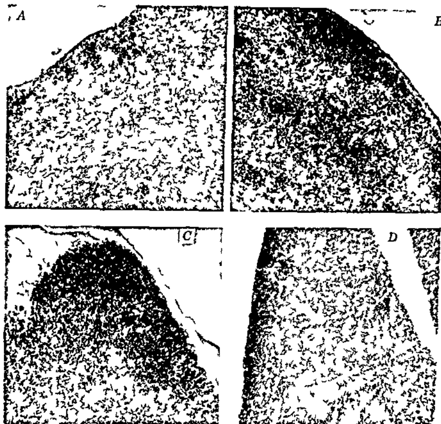



FIG 6 Injury and repair in the thymus after 300 r At 24 hours (A) the cortex has been largely emptied of small lymphocytes with pyknotic cells and nuclear debris accumulating in subcapsular macrophages By contrast an appreciable num ber of small lymphocytes persist in the medulla yielding a reversal of the usual staining intensity of the cortical and medullary zones By the third day (B) the necrotic debris has disappeared and a layer of large cells has begun to proliferate just beneath the capsule On the fourth (occasionally fifth) day (C) these cells have been transformed into immature large lymphocytes filling the entire cortex with an irregular boundary between cortex and medulla Pyknosis is often seen in the medulla at this stage By the fifteenth day the cortical lymphocytes have dif ferentiated to mature small forms and the architecture of cortex and medulla is almost normal again (Hematoxylin and eosin × 110)

70 days or more after irradiation (Table I) There was a striking difference in the pooled tumor incidence of the three series with a much greater yield of tumors in series C which had received 4 doses of 119 r each at 4 day intervals The differences are slightly exaggerated because

TABLE I

LYMPHOID TUMORS ENCOUNTERED AT SERIAL SACRIFICE OF C57BL MICE RECEIVING THE SAME TOTAL X RAY DOSE WITH FRACTIONATION AND PERIODICITY VARIED

| Time of sacrifice days after last x ray | Series A | Series B | Series C |
|---|---|---|---|
| 70 | — | 1/9 | 8/10 |
| 80 | 1/10 | 1/9 | 5/10 |
| 90 | 0/12 | 0/10 | 7/10 |
| 100 | 1/10 | 1/10 | 4/10 |
| Total | 2/32 (6%) | 3/37 (8%) | 24/38 (63%) |

Numerator = number of mice with lymphoid tumors denominator" = number sacrificed

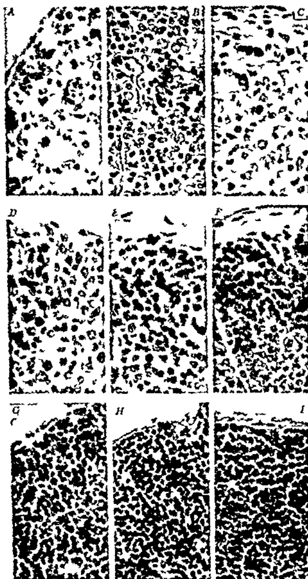
the anticipated latent periods for tumors arising in mice treated as in series A and B are greater than for series C

## Discussion

Confirming our earlier observations (9) periodic total body irradiation of C57BL mice at 4 day intervals elicited a much greater number of thymic lymphomas in animals sacrificed at 70 to 100 days than an equal dose given either on a daily fractionated sequence or as a single exposure The pattern of thymic weight response and the histological appearance during injury and regeneration in the irradiated thymus glands were essentially the same however after each of these schedules of irradiation Indeed, the degree of thymic injury was mildest, and the capacity for regeneration perhaps greatest in the intermittently irradiated animals Thus there is no apparent correlation between the severity of thymic radiation injury and subsequent lymphoid tumor yield This conclusion is consistent with other evidence (8 11) that these tumors arise by an indirect induction mechanism The lack of correlation may of course be attributable to the criteria employed since thymic weight and histology are admittedly crude indices of radiation injury

The weight curve of the irradiated thymus is nonetheless of interest particularly since it appears to differ distinctively from that of other irradiated lymphoid tissues In contrast to the slow recovery of lymph

FIG 7 Injury and repair in the thymus after 300 r Nuclear debris chokes the cortical macrophages at 24 hours (A) contrasting with the persistence of some small lymphocytes and absence of debris in the medulla (B) The debris is gone at 48 hours (C) leaving the cortex strikingly empty of lymphoid cells Surviving large epithelial reticular (?) cells beneath the capsule begin to proliferate on the third day (D) and then apparently transform into large immature lymphocytes on the fourth and fifth days (E and F) These immature cells then differentiate into progressively smaller more mature lymphocytes (G day 7) Mitotic figures are numerous at about 4 to 7 days and then decrease in frequency By day 15 (H) the cortex is repopulated by small lymphocytes and again closely resembles the normal thymic cortex (T) (Hematoxylin and eosin X 550)


A
B
C
D
E
F
G
H
I

node weight thymic weight rebounds vigorously to an abortive peak at about 9 to 15 days after irradiation only to fall sharply again for about 10 days There is then a more gradual but sustained recovery which may require as long as 70 to 80 days to reach the control baseline The secondary sharp decrease in weight is not associated with histological evidence of a new wave of cellular radiation damage It appears to be

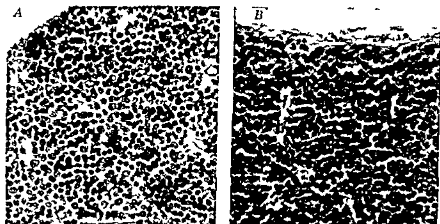



Fig 8 In occasional instances regeneration of the thymic cortex after irradiation proceeds through the stage of repopulation by immature lymphocytes but these fail to undergo further differentiation Here (A) large densely packed slightly hexagonal lymphocytes fill the cortex 30 days after 300 r and mitotic figures remain numerous (B) normal thymic cortex for comparison (Hematoxylin and eosin × 550)

a reasonably reproducible phenomenon in several experiments in this laboratory and is to be found in the data of an earlier study by Brecher *et al* (1) although not explicitly discussed in their report The explanation for this bizarre oscillation must await further experimental study It is interesting however that no secondary involutional phase occurs when thymic regeneration is promoted by spleen or thigh shielding or bone marrow injection (10)

## Summary

Strain C57BL mice of both sexes were given single or fractionated doses of total body x irradiation The irradiated mice and their untreated age controls were then sacrificed at a series of time intervals extending to 100 days after treatment The weights of the thymus spleen and pooled superficial lymph nodes were measured and histological examination of these tissues was made

Lymphoid tumors were encountered in the thymus glands of groups sacrificed 70 or more days after irradiation. Confirming an earlier report (9) the incidence of such tumors was much greater in mice given fractionated doses at 4 day intervals (63%) than in those receiving a single exposure (6%) or daily fractionated treatment (8%). No comparable difference in the form of the thymic weight curve in relation to technique of irradiation was observed, however, and the histologic changes in the three series were essentially similar. The histological sequence of events during radiation induced thymic involution and regeneration is described and illustrated.

A curious and apparently characteristic aspect of the postirradiation thymic weight curve was noted. Subsequent to the well known dramatic involution which occurs within the first few days, there was a sharp rebound toward normal levels, followed at about 15 to 20 days after treatment by a second fall to weight levels almost as low as those immediately after irradiation. This unexplained secondary fall was not observed in the weight curves of the irradiated spleen or superficial lymph nodes. Ultimately there was a more gradual but sustained recovery of thymic weight which required as long as 70 to 80 days to reach control levels.

## REFERENCES

1 Brecher, G., Endicott, K. M., Gump, H., and Brawner, H. P. *Blood* 3, 1259 (1948)
2 Carnes, W. H., and Kaplan, H. S. *Federation Proc* 15, 726 (1956)
3 Carter, R. E., Harris, P. S., and Brennan, J. T. Los Alamos Scientific Laboratory Report LA 1075 (1950)
4 Deanesly, R. *Quart J Microscop Sci* 72, 247 (1929)
5 Harris, P. S., and Ellinwood, L. F. Los Alamos Scientific Laboratory Report LA 1629 (1953)
6 Kallmann, R. F., and Kohn, H. I. *Radiation Research* 2, 280 (1955)
7 Kaplan, H. S. *J Natl Cancer Inst* 11, 83 (1950)
8 Kaplan, H. S. *Cancer Research* 14, 535 (1954)
9 Kaplan, H. S., and Brown, M. B. *J Natl Cancer Inst* 13, 185 (1952)
10 Kaplan, H. S., Brown, M. B., and Paull, J. *J Natl Cancer Inst* 14, 303 (1953)
11 Kaplan, H. S., Hirsch, B. B., and Brown, M. B. *Cancer Research*, in press
12 Kaplan, H. S., Nagareda, C. S., and Brown, M. B. *Recent Prog Hormone Research* 10, 293 (1954)
13 McEndy, D. P., Boon, M. C., and Furth, J. *Cancer Research* 4, 377 (1944)
14 Murray, R. in "Histopathology of Irradiation" (W. Bloom, ed.), p. 446, McGraw Hill Book Company, New York, 1948
15 Rask Nielsen, R. *Brit J Cancer* 3, 549 (1949)
16 Stroud, A. N., Gurian, J. M., Brues, A. M., and Summers, M. M. *Radiation Research* 2, 267 (1955)

# 10

# Cytocidal Action of X Rays on Lymphocytes According to Time Lapse Cinemicrography and Unstained Cell Counts

ROBERT SCHREK

Tumor Research Laboratory Research Service
Veterans Administration Hospital Hines Illinois

As radiation is an important factor in modern life we want to know as much as we can about its effect on biological systems But there is another reason for using x rays in research X rays are a wonderfully delicate tool that can do a better dissection of a cell than Chambers ever dreamed of If we are going to use x rays as a cytologic tool it is important to keep it delicate i e to keep the dosage low What I have to say in regard to radiation applies only to relatively low dosages 50 to 1000 r

Let us start with those findings which have been accepted by workers in the field First all workers agree that x rays in small doses kill lymphocytes This fact was suspected in animal studies in view of the known radiosensitivity of lymphocytes The fact was demonstrated in *vitro* by the method of unstained cell counts In this method a suspension of lymphocytes is prepared usually from the thymus of the rat or rabbit When eosin or safranine is added to this suspension nearly all the cells are unstained because living cells are impermeable to the dye An occasional cell is stained and these are considered dead cells The living cells can then be counted in an ordinary hemocytometer When *the suspension is radiated with 1000 r* and incubated at 37 nearly all the cells are seen to be dead and are stained readily with eosin An occasional cell is still resistant to staining and these unstained cells are usually seen to be cells other than lymphocytes By a periodic counting of radiated and nonradiated cells survival curves can be obtained By *this method it can be said that the normal lymphocyte survives on the* average about 22 hours the lymphocytes radiated with 140 r survive only 11 hours and after 1000 r the cells survive only 6 hours (10)

The finding that x rays kill lymphocytes is in accord with the reports of many workers Patt *et al* (8) proved it by unstained cell counts Trowell (12) showed it in tissue culture by counting the number of pyknotic cells in histologic preparations Other workers have shown the same thing *in vivo* Dougherty and White (2) found that x rays caused the regression of lymphoid tissues even in adrenalectomized rats Ross *et al* (9) collected the cells from the thoracic duct in rats and dogs and found that the lymphocytes start to degenerate 3 hours after radiation of the animals

It may be concluded that x rays in small doses kill lymphocytes both *in vitro* and *in vivo*

The next fact that seems to be accepted is that the death of the irradiated lymphocyte is due to or is associated with the development of intranuclear vacuoles This is most dramatically demonstrated by means of time lapse cinemicrography (11) A suspension of rabbit lymphocytes was irradiated with 1000 r *in vitro* and photographed during incubation Photographic prints made from the cinemicrographic film showed that most lymphocytes appear normal 3 hours after irradiation The nuclei of the cells have large dark chromatin masses and some cells in the projected film are in ameboid motion At irregular intervals individual cells develop small intranuclear vacuoles although the rest of the nucleus still appears to be normal in structure and the cell itself may still be in ameboid motion in the projected film A few minutes after the development of the intranuclear vacuole the nucleus and then the cell as a whole becomes irregular in shape In the projected film the cell and the nucleus are seen to change shape rapidly with the development of multiple lobules The nucleus also develops lobules which may separate from each other forming two or more nuclear masses The original small vacuole meanwhile has enlarged rapidly and the chromatin has been pushed peripherally forming a thin chromatin ring With the continued enlargement of the vacuole the chromatin ring breaks and forms a horseshoe shaped mass then a crescent and finally a dark round chromatin mass The cell usually has two or more lobules Each lobule contains a part of the nucleus with an intranuclear vacuole and a separate chromatin ring and each ring ruptures giving rise to a small dark chromatin mass In this way the cell ultimately develops a fragmented pyknotic nucleus

I first saw these abnormal degenerating cells while making unstained cell counts of irradiated lymphocytes Cells with vacuolated nuclei in irradiated animals were however described as early as 1907 by Warthin (14) More recently Ross *et al* (9) described vacuoles in the lympho

cytes that they collected from the thoracic duct of irradiated animals Warren *et al* (13) demonstrated intranuclear vacuoles in irradiated tissue which were fixed by the modern techniques of freezing and drying

The next finding which I wish to report has not as yet been confirmed Normal nonirradiated lymphocytes were studied by time lapse cinemicrography It was surprising to find that they also develop intranuclear vacuoles and lobules Morphologically the changes are the same in the irradiated and nonirradiated cells although the rates of death are quite different Therefore I prefer to say that x rays accelerate the rate of death rather than that they kill lymphocytes

The findings can be summarized by the following biological equations



$$Ln \rightarrow Lx \rightarrow Lv \rightarrow Lp \quad \text{in irradiated cells}$$
$$Ln \rightarrow Lv \longrightarrow Lp \quad \text{in nonirradiated cells}$$

The normal lymphocyte is represented by *Ln* After irradiation the cell is morphologically the same but physiologically altered and is represented by *Lx* This altered cell on incubation at 37 soon develops into a cell *Lv* with an early intranuclear vacuole and finally into a cell *Lp* with a pyknotic nucleus

The nonirradiated lymphocyte also develops a vacuolated and then a pyknotic nucleus The vacuolated cells in the irradiated and the nonirradiated suspension seem to be similar and both are therefore given the same symbol of *Lv* In the same way *Lp* is used to represent the pyknotic cell both in the irradiated and the nonirradiated suspension

The biological equations can be studied as if they were chemical equations It is necessary to study the properties of each component the rates of each reaction and the effect of various reagents on the rates of reaction

The normal cell *Ln* and the cell immediately after irradiation *Lx* have nuclei which are filled with small bright stationary granules according to darkfield microscopy Both cells are viable as seen by their resistance to staining with eosin by their rhythmic movements and by occasional ameboid movement in time lapse cinemicrography

The vacuolated cell *Lv* resists staining with eosin and as far as this criterion is concerned the cell may be considered viable The ring surrounding the intranuclear vacuole stained with Feulgen stain and is evidently derived from chromatin material The vacuole itself however failed to stain with any of the histochemical stains used With darkfield illumination the vacuole appears dark and the ring bright

The pyknotic cell *Lp* owing to irradiation or normal death, is of course definitely dead and takes the eosin stain The chief characteristic of the nucleus is its uniformity in appearance with phase microscopy and

with stains The nucleus is stained by Feulgen's method With dark field microscopy it is found to be optically empty Furthermore on pressure it can be expressed from the cell in small drops On centrifugation the pyknotic nuclei of two or more cells fuse to form one dark vacuole The pyknotic nucleus seems then to be a viscid structureless fluid which is derived from the chromatin material of the original nucleus

Now we can proceed to the quantitative aspects of the various reactions The rapidity of the change produced by irradiation from a normal lymphocyte to a physiologically altered cell ($Ln \longrightarrow Lx$) cannot be determined directly but from other evidence it would seem that this reaction is rapid and occurs during the process of irradiation and for a few minutes afterward

The onset of vacuolization can be determined directly by time lapse cinemicrography or indirectly by the method of unstained cell counts After 1000 r the reaction $Lx \longrightarrow Lv$ takes on the average about 5 7 hours and after 140 r about 10 7 hours These durations represent medians as individual irradiated cells may develop vacuoles within an hour after irradiation and other cells may remain normal for many hours

The period of lobulation in irradiated cells $Lv \longrightarrow Lp$ was difficult to determine owing to the uncertainty of the exact onset of vacuolation and pyknosis The period was defined as the average time between the onset of the rapid changes in the shape of the nucleus to the time of the rupture of the chromatin ring The period of lobulation was about 20 minutes

In nonirradiated cells the median duration of the process $Ln \longrightarrow Lv$ is 21 7 hours The average period of lobulation $Lv \longrightarrow Lp$ is 20 minutes The period of lobulation is approximately the same in the irradiated and the nonirradiated cell although not enough cells have been studied to rule out any minor differences

Of considerable interest is the effect of various factors on these reactions Some of the factors have been reviewed by Patt (7) The change of $Ln$ to $Lx$ occurs at any temperature from 4 to 41 There is no temperature where the reaction is maximum and there is no temperature which inhibits the reaction

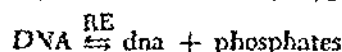
The effect of the pH on this reaction has not been studied to any great extent although there is some evidence that large variations in pH have no effect on the change of $Ln$ to $Lx$ Oxygen tension during irradiation and shortly thereafter is important however For maximum effect the suspension has to be equilibrated with an atmosphere of 20 to 100% of oxygen Lowered oxygen tension and particularly anoxia at 37 partly inhibit the reaction so that less $Lx$ cells are formed and the

*lymphocytes seem to be radioresistant* The reaction *Ln* ⟶ *Lx* is also partly inhibited by the addition of 20 mM of cysteine at 37 shortly before irradiation

The transformation of the irradiated cell to the *vacuolated cell and* the pyknotic cell *Lx* ⟶ *Lv* ⟶ *Lp* also depends on various conditions The reaction takes place when the cells are incubated at 21 to 41 with the maximum reaction occurring at 37 The reaction is completely inhibited at 4 to 17 so that the irradiated cell *Lx* survives *as long as the nonirradiated cell at these temperatures Furthermore the* pH of the incubated irradiated suspension has to be between 6 5 to 8 5 although no pH is optimum for the reaction *Lx* ⟶ *Lp* At pH of 6 0 the reaction is completely inhibited and the irradiated cells survive as long as the nonirradiated cells The effect of oxygen tension has not yet been studied on this reaction as far as I know Cysteine has no effect so that addition of this compound after irradiation does not protect the irradiated cells

It should be *noted* that *during irradiation anoxia or cysteine at 37* partially protected lymphocytes from irradiation but after irradiation the cells were protected from an early death by the addition of acid to a pH of 6 0 or by incubation at a low temperature

What does it all mean? Any answer to this question is speculation We have seen that the normal nucleus became a pyknotic nucleus plus one or more vacuoles This reaction occurred spontaneously and was accelerated by irradiation The reaction seemed to be highly radiosensitive This *morphologic* change *seemed* to *involve the chromatin* material The normal chromatin became the altered chromatin of the pyknotic nucleus plus the vacuolar substance which could not be stained by any *of the histochemical stains tried These* and other findings suggest the following equation as a working hypothesis



$$\mathrm{DNA} \overset{\mathrm{RE}}{\rightleftarrows} \mathrm{dna} + \text{phosphates}$$

The reasons for formulating this reaction cannot be given here but I shall try to explain the equation In the degenerating lymphocyte the deoxyribonucleic acid (DNA) in the chromatin is assumed to produce the depolymerized deoxyribonucleic acid (dna) plus the vacuole which *is assumed to consist of inorganic phosphates On the other hand the* normal lymphocyte is known to take up phosphates and produce DNA This synthesis is assumed to be controlled by an enzyme RE The equation states that in the normal lymphocyte both reactions occur reversibly with the enzyme RE controlling only the synthesis If RE is assumed to be highly radiosensitive radiation will inhibit the synthesis of DNA and the reverse reaction can proceed unhampered In the nonirradiated

lymphocyte RE deteriorates spontaneously and the same type of degeneration occurs According to this hypothesis death of the irradiated animal may be due to the lack of RE or of some essential DNA Injection of bone marrow or spleen might replace these compounds

It should be observed that this hypothesis combines the hypotheses and findings of other workers It is in accord with the work of Hevesy (5) and Klein and Forrsberg (6) that irradiation decreases the uptake of $P^{32}$ and inhibits the synthesis of DNA It is in accord with the studies of Harrington and Koza (4) and others that x rays cause the depolymerization of DNA Barron *et al* (1) suggest that x rays cause the inactivation of an enzyme and are especially destructive to phosphatases in solution Finally the liberation of phosphates would disturb the osmotic pressure and cause the influx of water into the nucleus And Fulla (3) claims that radiosensitivity is dependent on a disturbance of the osmotic pressure in the nucleus

The equation is a hypothesis but we still have to see if it works

In conclusion we may say quite definitely that x rays kill lymphocytes or rather that x rays decrease the longevity of the lymphocyte This reaction occurs both *in vitro* and *in vivo* Second it is quite definite that the death of the irradiated cell is due to the development of intranuclear vacuoles The vacuoles also occur in nonirradiated lymphocytes These findings suggest that there is a specific biochemical reaction that is radiosensitive The reaction occurs in lymphocytes and possibly also in mitotic cells but not in other cells This biochemical radiosensitive reaction may involve a reversible anabolic catabolic reaction of DNA

REFERENCES


1 Barron E S G Dickman S Muntz J A and Singer T P *J Gen Physiol* 32 537 (1948 1949)

2 Dougherty T F and White A *Endocrinology* 39 370 (1946)

3 Fulla G *Am J Roentgenol Radium Therapy* 44 649 (1940)

4 *Harrington N J and Koza R W Biol Bull* 101 139 (1951 )

5 Hevesy G *in* Radioactive Indicators Their Application in Biochemistry Animal Physiology and Pathology Interscience Publishers New York 1948

6 Klein G and Forrsberg A *Exptl Cell Research* 6 211 (1954)

7 *Patt H M Ann N Y Acad Sci* 59 649 (1955)

8 Patt H M Blackford M E and Straube R L *Proc Soc Exptl Biol Med* 80 92 (1952)

9 Ross M H Furth J and Bigelow R R *J Hematol* 7 417 (1952)

10 Schrek R *Radiology* 46 395 (1946)

11 Schrek R *Radiology* 65 912 (1955)

12 Trowell O A *J Pathol Bacteriol* 64 687 (1952)

13 Warren S Holt M W and Sommers S C *Am J Clin Pathol* 22 411 (1952)

14 Warthin A S *Am J Med Sci* 133 736 (1907)

# Added Comment

L O JACOBSON
Argonne Cancer Research Hospital and the Department of Medicine
The University of Chicago Chicago Illinois

The work I wish to mention briefly is related to the research I have been doing during the last few years—namely the recovery of animals from radiation injury as a result of spleen shielding or spleen transplants or after the intraperitoneal or intravenous administration of homogenates of spleen bone marrow etc

My impression over the first few years was that irradiated animals given tissue of hematopoietic origin lived because they probably were getting from the administered cells a humoral substance that made it possible for their own hematopoietic tissue to regenerate This theory was supported to my way of thinking by the work of Lorenz and others and by some data of our own that indicated that heterologous material of hematopoietic origin was effective For example rat marrow was effective in mice We have shown that mouse marrow mouse spleen and mouse liver are effective in the rabbit

There is work in progress however in various laboratories (not our own) which should be brought to your attention The studies I shall discuss demonstrate that we must change our thinking somewhat about what cells can do in a new host

Barnes and Loutit working in England did the experiment to be described They irradiated mice and then injected into them marrow from the same strain but with chromosome 6 marked They found that the mice survived the radiation A very large percentage of the make up of the surviving animal s hematopoietic tissue at intervals after irradiation was indeed made up of donor cells as indicated by a study of the marked chromosomes

Lindsley and Odell at the same time did a beautiful piece of work Within the same strain of rat they had a group with a type C blood and another group with type D They irradiated the type D animals with a

lethal dose and injected type C bone marrow In 120 days 85% of the red cells in the circulating system of the animal was from the donor

There is an interesting report by Dr L Cole in a recent Navy bulletin to my knowledge not yet in the open literature He used the technique of Gomori developed some years ago that demonstrates that mouse and rat cells are different from the point of view of alkaline phosphatase staining Dr Cole irradiated mice and gave them rat cells As you know mice will live after lethal irradiation if given rat cells Then he simply stained the cells and the bone marrow and found that at 20 days after irradiation the bulk of the cell population was alkaline phos phatase positive This of course tends to indicate that the rat cells are living in the mouse and may well be responsible for the observed survival In his publication Cole says that he observed some of his animals as late as 120 days after irradiation and that alkaline phos phatase positive cells still existed

If one gives normal CF1 mice DBA leukemic cells (work done by Dr E L Simmons in our own laboratory) no leukemia develops CF1 mice will develop leukemia however if they are irradiated prior to being given DBA leukemic cell preparations On the other hand a normal CF1 mouse which is irradiated with its spleen shielded will not develop leukemia even when given DBA leukemia if the spleen is re moved shortly after irradiation If the CF1 mouse is x irradiated and given normal CF1 cells and nonleukemic DBA cells so that it survives the radiation and then after 1 month it is given leukemic DBA the mouse will develop leukemia and die

In summary the problem of the mechanism of recovery of irradiated rodents after spleen shielding or the injection of hematopoietic tissue is not settled It now appears that my original contention that the shielded or injected tissue brought about recovery largely by a humoral substance or group of substances is probably wholly or partly wrong since it has been demonstrated that the injected tissue multiplies and repopulates the depleted hematopoietic tissues of the irradiated animal One cannot entirely discard the humoral theory at the present time how ever because one would still have to explain the recovery of the capacity to form antibodies and other phenomena that cannot be explained by simple repopulation For example the positive alkaline phosphatase reaction observed in irradiated mice that have received rat marrow by injection may conceivably be a more complicated problem than a matter of simple repopulation

# Added Comment

JACOB FURTH

Children's Cancer Research Foundation Inc Children's Hospital
Boston Massachusetts

I shall not attempt to discuss any of the papers just presented In stead I propose to correlate observations on human beings with experimental studies on the induction of leukemias in animals by ionizing irradiation

The splendid presentations on mouse leukemia given by several of my colleagues and Professor Watts excellent review of human leukemias induced by ionizing irradiation enable me to be brief

To begin with I shall have to repeat what Professor Watts has said There is only a single known agent well documented to cause leukemia—ionizing irradiation But ionizing irradiation probably accounts for only a very small percentage of leukemias The situation is somewhat analogous to that of lung tumor incidence Both appear to be on the increase With both an agent is implicated which is responsible for some but not for most of the increase

Professor Watts reviewed the three sources of information available in man—the radiologists the Japanese (Hiroshima) casualties and the therapeutically induced leukemias that is therapeutic accidents

I wish to repeat the generalization that leukemia is not a single disease The situation can be understood only if we consider the various types of leukemias separately their incidence in a given population in relation to age sex occupation genetics and other factors which may be involved in their genesis

With respect to leukemias among radiologists as far as I can gather from the statistics the ratio of the two main types myeloid and lymphoid is about the same as that in the population at large The Hiroshima cases were almost all of the myeloid or "blast type but so are most leukemias reported in the Japanese population One wonders whether this is due to genetics or to some other factor which favors the induction of myeloid leukemia At present we have but meager information on the relation of myeloid stimulation to leukemogenesis

The Hiroshima story is not yet final The reported decline proved to be temporary and very recent information indicates that leukemia is still occurring in fairly large numbers as if a new peak will make its appearance Will some of these leukemias be of the lymphoid type? Different types of human leukemias have different peaks and the full story cannot be told until final mortality statistics of this generation are available

Turning to therapeutic accidents we have two interesting groups of well analyzed cases In England (Court Brown and Abbatt) spinal column radiation of young adults for rheumatoid arthritis caused mainly myeloid leukemias In the United States the data of Simpson Hempelman and Fuller showed that in children treated with x rays for thymic enlargement the type of leukemia produced was lymphoblastic or of the stem cell—that is of the childhood type It is important to note that localized irradiation can induce lymphoid leukemias in man (in mice the importance of total body irradiation is stressed) and that the leukemogenic dose was small in children (somewhere in the range of 150 to 400 r) and high in adults (thousands of roentgens)

One wonders whether these differences are due to the site of the radiation or to age at time of irradiation The Court Brown and Abbatt cases relate to spinal column that is bone marrow irradiations those of Simpson *et al* to predominant exposure of lymphoid tissues Are sites of the radiation and age determinants of the type of leukemia induced?

The incidence of leukemia in both man and animals is proportional to the dose The maximum induction rate both in Japan and among patients irradiated with rheumatoid arthritis is close to 1 in 100 exposed people a developmental rate of several hundredfold of that in the non irradiated population Animal experiments are carried out on such small scale that neoplasia induction rates in these ranges would not be registered This may explain why rats rabbits and guinea pigs in which irradiation studies were conducted were declared resistant species The extraordinary sensitivity of mice to leukemias is remarkable No comparative figures are available but it seems that the leukemia inducing tissue dose was much smaller in the Hiroshima (total body irradiated) than in the arthritis (partial body irradiated) cases suggesting the role of some protective influence

In mice the type of leukemia most thoroughly investigated originates in the thymus the other types have not been adequately studied Frequently generalizations are made from these experimental studies in mice on the induction of lymphomas of thymic origin as if the results

would hold true for all leukemias of all species It was shown a long time ago that removal of the thymus will prevent development of most *leukemias in certain strains of mice* because most of them are thymic in origin Thymectomized animals lived longer but other types of leukemias killed many of them at later age

If thymectomized mice are irradiated *other* types of leukemias come into prominence In the Bf strain both lymphoid and myeloid leukemias occur in high percentage after irradiation The spontaneous incidence of leukemia in this strain is low the lymphoid is more common and its peak occurrence precedes that of myeloid leukemia Thymectomy prevents the induction by ionizing irradiation of lymphoid leukemias of thymic origin but not of myeloid leukemias The frequency of myeloid leukemias actually rises because thymectomy prevents the thymic type of lymphoid leukemia (Upton and Furth)

Briefly the factors involved in leukemogenesis of myeloid and nonthymic lymphopoietic tissues *should not be identified with those of* thymic lymphoma of mice *I have singled out two types of experimental* leukemias thymic lymphoma and myeloid leukemia We have but scant *information on chronic myeloid leukemia with maturation* and we have no equivalent in mice of the chronic small cell type of lymphoid leukemia of man We are uncertain as to the relationship between small celled lymphosarcoma of man and large celled thymic lymphosarcoma of the mouse

Generalized nonthymic leukemias occur in a fair degree of frequency in some strains of mice This has been thoroughly studied by Kirschbaum and Law Law found that in some inbred strains almost all leukemias are thymic and in other strains almost all are extra thymic The many available inbred strains of mice provide rich material for the study of both genetic factors and responsiveness to extrinsic agents

There is no evidence to indicate that a virus as described by Gross plays a significant role in the development of leukemia after ionizing irradiation because the irradiation in appropriate doses will induce leukemia in a very high percentage of mice of almost every low leukemia strain whereas this hypothetical virus has a high strain selectivity and infects only newborn animals This leads me to problems on fractionation *Fractionating* a given total dose can enhance the leukemia incidence in a low leukemia strain of mice from a few per cent to as high as over 70% (Kaplan *et al*) There has not been enough experimentation on the role of timing in fractionation of a given dose The interval as well as the size of the dose is of major importance By proper extension of the interval the leukemia induction rate can be reduced *There is some evidence indicating that the effects of fractiona*

tion are not the same for different types of leukemias More research is needed along these lines

Differences in hormonal factors on leukemia induction are worthy of a few comments In most strains of mice estrogens are leukemogenic (Gardner) Leukemia occurs with greater frequency in females and is of the lymphoid type Androgens are antileukemogenic This is not true in man Most leukemias in man are myeloid and the frequency is greater in males

In a large scale study of the late effects of an atomic explosion ( Operation Greenhouse ) in the groups exposed to more than 700 r thymic lymphoma occurred with greater frequency among males and the threshold dose was about 500 r In females the threshold dose was lower even the 192 r group showing a distinct increase The earliest cases appeared in the fourth month postirradiation and the peak incidence occurred at 7 to 12 months after exposure The greater incidence of thymic lymphomas in males is contrary to earlier experience with most strains of mice exposed to sublethal doses in which these tumors were more common in females Other forms of leukemia including generalized and other lymphomas reticulum cell sarcomas and rarely myeloid leukemias occurred relatively late in life and were less numerous in the irradiated animals than in the controls After correction for longevity however the incidence of these types of leukemia was slightly higher among the exposed mice (Upton Furth *et al* ) In this strain (LAF1) unlike most other strains of mice ovariectomy in females greatly enhances the leukemia incidence (Buffett and Furth) Thus in this strain there is an androgenic predominance in the induction of lymphoid leukemias

Leukemias do occur in castrates in both males and females and the only permissible generalization is that gonadal hormones are modifiers of leukemia incidence but not its determinants Corticoids influence the development of spontaneous lymphoid leukemias and their induction by x rays but it is doubtful that they affect myeloid leukemias of mice and it is unknown what role if any corticoids play in the development of spontaneous leukemia of man and animals

I should like to say a word about the meaning of the terms dependence and responsiveness In animal experimentation autonomy can be well defined an autonomous tumor is that which grows in an untreated host But autonomy is not synonymous with responsiveness Some autonomous tumors (grown in normal hosts) are resistant and others are sensitive to the physiologic forces which influence proliferation of that particular type of cell Recently we have learned to distinguish three types of autonomous tumors (*1*) those responsive to their physiologic

restraining force (2) those stimulated by them and (3) those indifferent to them One may theorize that leukemias of the same types probably exist (that is dependent autonomous of either of the three variant types) but thus far only autonomous leukemias of unspecified character have been studied in animals

I should like to point to a few interesting problems related to leukemogenesis which deserve clarification Research on protective factors was initiated by Dr Jacobson when he demonstrated the protective effect of splenic shielding Later the findings of Lorenz Jacobson Kaplan and their associates turned attention to bone marrow There are reports that other organs may also contain protective agents Is this protective ability a universal property of all cells or is it a special feature of marrow cells or is it present in greater concentrations in some cells than in others? Only future research will tell That there is a protecting substance circulating in the blood has long been shown by parabiosis studies the more recent demonstration of radiation protection (including prevention of leukemia) by injection of hemopoietic tissues discussed by Dr Jacobson opens up a highly fertile field of research in radiation biology

The counterpart of this radiation protecting agent is the leukemia producing agent created by irradiation which was discovered by Dr Kaplan This is one of the most spectacular developments of the past few years Does this factor affect other types of leukemias as well or is it related only to the highly hormonal dependent thymic lymphoma? Dr Kaplan with whom I have been discussing this problem is almost willing to generalize and to postulate that most perhaps all neoplasms are induced by an indirect mechanism I shall cite some recent observations relevant to this problem

In a current unpublished study (Buffett and Furth) undertaken primarily to learn about pituitary tumorigenesis a group of animals was irradiated over the lower two thirds of the body the thymus and head being shielded In another group the head and neck were heavily irradiated and lower parts of the body were shielded The leukemia incidence in the partially irradiated groups dropped to almost zero Other neoplasms occurred in the irradiated areas at greater frequency however Indeed it is possible to raise the local tissue dose by partial body irradiation increasing thereby the number of neoplasms arising at radiation sites It seems that the circulating leukemia inducing and leukemia protective agents are not or are only very slightly effective against other neoplasms Apparently protective and promoting influences of neoplasia induction have to be considered individually with each neoplasm A neoplasm induced by hormonal derangement can be prevented

Homogenates of spleen after total body irradiation contain very little or none of this inhibitor of deoxyribonuclease If the radiation acts by destroying the inhibitor the nuclease would be free to depolymerize the DNA and this reaction would have the characteristics of temperature and pH dependence such as Dr Schrek described

We have attempted to generalize from this hypothesis and to suggest that the cell death which follows irradiation may be secondary to the destruction of the inhibitor and th consequent depolymerization of the cells by the deoxyribonuclease and that the recovery from irradiation may be associated with a compensatory excess increase in this inhibitor and this might then give rise to tumor development and leukemia

DR ROGER KLEIN (Galveston Texas) Perhaps you have read in the newspapers recently the story of the twin girls in Michigan both of whom had leukemia There are in the literature other cases of leukemia in twins A very interesting statistic by Macklin in the *Journal of Heredity* in 1940 shows that when monozygous twins both had cancer in 95% of the cases the cancers were of the same tissue

This fact seems to prove that carcinogenesis is related to the problem of cell differentiation The beautiful work by Dr Kaplan and the most recent publications in radiology and endocrinology show that the mechanism of carcinogenesis is indirect, probably in two steps We can understand it by the progressive and finally irreversible loss of a factor of inhibition and of maturation which is linked with the species identity and the multiplication of a factor of proliferation which is bound with the tissue specificity

By the same mechanism of acquisition of autonomy by loss we could perhaps understand why the autonomous cancers are homotransplantable and not the normal tissues Both factors probably are involved in the mechanism of protein synthesis of antibody synthesis and of immunity and that could explain that cancer is also a loss of the mechanism of cellular immunity

DR HENRY S KAPLAN (San Francisco California) I should like to comment on a few items that have come up in relation to the many challenging things that other speakers have presented this afternoon

Starting first with Dr Hauschka s presentation you will recall that I demonstrated a lymphoma which arose in a thymic graft in a nonirradiated mouse We have been conducting serial biopsies of some of these tumors allowing the same tumor to grow back between biopsies and ultimately of course when the tumors become disseminated taking a final biopsy at the time of autopsy

At the time of each biopsy we have preserved a piece of tissue for histological examination permitting us to establish and verify the diagnosis of lymphosarcoma at each of the biopsy stages and we have then taken the remainder of the tissue and inoculated it either intraperitoneally or subcutaneously into young normal C57 black mice Unfortunately we are still in the 16-gauge trocar stage Dr Hauschka Nonetheless it would appear that there is a progressive increase during the natural history of these tumors in their capacity to "take in mice of the same strain

This appears to me to be a counterpart of Dr Furth s demonstration with the transplantable pituitary tumors of a possible conditioned relationship to the host of origin. *Thus it would appear if these observations are confirmed as we extend them* that there may be (in the case of the lymphoma developing *in situ*) a conditioned state of transitory nature

## Part IV

# The Leukemias and the Malignant Lymphomas

Chairman
**Carl V Moore**
School of Medicine
Washington University
St Louis Missouri

# The Leukemias — Their Scope

MAURICE N RICHTER

Department of Pathology of the College of Medicine and the University Hospital New York University—Bellevue Medical Center New York New York

In a symposium on leukemia there should be a discussion of the nature of leukemia the various conditions that appear to be related to it, and the scope of discussions pertaining to the disease

An attempt to define leukemia would naturally fail A definition based on morphological features would not satisfy a biochemist nor would his definition be considered suitable by a geneticist a biologist or a clinician We need however some idea of the kind of disease with which we are working We also need to define more clearly the boundaries between leukemia and the conditions closely related to it, and conditions that are less closely related though they may resemble leukemia

An example may be noted in the general group of malignant lymphomas It is now widely believed that lymphatic leukemia and lymphosarcoma are different manifestations of the same disease Acceptance of this view has come about largely from the experimental study of leukemia in mice As has already been reported each of the characters studied—biologic genetic morphologic and immunologic characters transplantability etc—has its counterpart in a similar character of tumors other than leukemia Moreover in transmission experiments certain features of the disease may be modified by the method of inoculation or by modifying the immunological background of the host

Less obvious are the relationships of Hodgkin's disease reticulum cell sarcoma and mycosis fungoides in which there has been no opportunity for experimental transmission of these diseases in laboratory animals

A closer relationship has been noted among the proliferative diseases of the reticuloendothelial system among which have been described

As stated above monocytic leukemia is often described as a proliferative disorder of the reticuloendothelial system There has however long been a tendency to recognize two varieties only one of which is so related the other arising from primitive cells of the bone marrow Without reviewing the literature in detail it may be stated that the clinical hematological and pathological features of the two types have been described in detail especially by Dameshek (2) Downey (3) Watkins and Hall (7) Belding *et al* (1) and others Nevertheless there continues to be uncertainty regarding the degree of reticulum and reticuloendothelial proliferation (6)

An opportunity was afforded to supplement available material with cases of monocytic leukemia from the Armed Forces Institute of Pathology These cases were studied particularly by section methods to determine to what extent this method afforded evidence for participation of the reticuloendothelial system

Forty five cases in which the diagnosis of monocytic leukemia was made were found suitable for study The changes observed on histological examination corresponded to those reported in the literature but the writers interpretation of some of the findings was somewhat different Attention centered on the relation of leukemic cells to the endothelium and reticulum Hypertrophy of the lining cells of the sinuses of lymph nodes and spleen was not uncommon and reactive mononuclear phagocytic cells arose therefrom These cells were not leukemic cells however and did not resemble them

Not infrequently leukemic cells were found in close proximity to the lining of the sinuses This however was not considered evidence of origin from the endothelial cells Numerous examples of ameboid activity with passage of the cells through the sinus walls suggested that proximity to the wall may be one stage in the process of such passage

It was also noted that even when hypertrophy of endothelial or reticulum cells occurred it was not widely distributed and did not involve all parts of the so called reticuloendothelial system There was no question of a "system" disease therefore regardless of the interpretation placed on these histological findings

As previously mentioned two types of monocytic leukemia have been described As the cases studied were not selected in a manner that would influence the type of disease it is reasonable to suppose that both types were represented In none of the cases however could it be established that reticuloendothelial cells played an important part in histogenesis Study of smears as well as sections showed that in many of the cases there were myelocytes in small numbers There were also

both localized and generalized forms the latter often with leukemic blood pictures In this connection one must recall the emphasis that has been placed on the reticuloendothelial system as a source of blood cells in the normal individual as well as the origin of cell accumulations in the leukemias The most direct association of leukemic cells with reticulum or reticuloendothelial cells has been postulated in monocytic leukemia a situation that may be anticipated from the widely accepted belief that monocytes are normally derivatives of the reticuloendothelial system The potentialities of the system" are not considered to be so limited however for the possibility of differentiation into other types of blood cells has been advanced to explain myeloid metaplasia and lymphocytic infiltrations

On this basis all the conditions under discussion have by one author or another been related to each other as different developmental orientations of the *reticuloendothelial system The views expressed regarding* the part played by the reticuloendothelial system are widely divergent however The differences may be illustrated by three quotations

The first is from a paper by Wiseman (8) in which he states Monocytic leukemia is one form of reticuloendotheliosis The varied reactions in monocytic leukemia suggest that all forms of leukemia may be hematologic varieties of reticuloendotheliosis

The second quotation is from Forkner (4) "The reticuloendothelial system which is really nothing more than differentiated mesenchymal macrophages must not be confused with primitive undifferentiated mesenchymal cells Many workers have placed entirely too broad an interpretation on the reticuloendothelial system thus causing it to lose its identity "

The third is from a paper by Gall (5) "It is proposed therefore that there is no such entity as a reticulum cell and that reticulum cell sarcoma as recorded in the literature consists of a group of conditions related only in that they represent primary neoplasms of hemopoietic tissue

We thus have views varying from complete acceptance of reticuloendothelium as the cell type of origin of all the conditions through degrees of doubt that the "system is responsible for all that has been ascribed to it to the final denial of its existence

In this situation one cannot accept any of the views expressed without reservation Indeed it might be better to start anew with a reinvestigation of the so called reticuloendothelial system—its various morphological components the reactions of its cells their relations to each other and potentialities for differentiation

As stated above monocytic leukemia is often described as a prolifera tive disorder of the reticuloendothelial system There has however long been a tendency to recognize two varieties only one of which is so related the other arising from primitive cells of the bone marrow Without reviewing the literature in detail it may be stated that the clinical hematological and pathological features of the two types have been described in detail especially by Dameshek (2) Downey (3) Watkins and Hall (7) Belding *et al* (1) and others Nevertheless there continues to be uncertainty regarding the degree of reticulum and reticulo endothelial proliferation (6)

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cases in which transition between cells resembling myeloblasts and monocytes suggested that the leukemic cells arose from primitive cells of the bone marrow

There are obviously other methods of examination that may give more and better information about the potential activities of reticuloendothelial cells than the one used in this study Failure to recognize formation of leukemic cells from the reticuloendothelial system does not indicate that such transformation may not occur nor does it disprove interrelationships between leukemias and other conditions It does however raise the question of the importance of the "system as an hematopoietic organ and indicates the need for continued investigation of the nature of the relationships involved

## REFERENCES

1 Belding H W Daland G A Parker F Jr *Cancer* 8 237 (1955)
2 Dameshek W *Arch Internal Med* 46 718 (1930)
3 Downey H in Handbook of Hematology (H Downey ed ) Vol 2 p 1275 Paul B Hoeber New York 1938
4 Forkner C E in "A Symposium on the Blood and Blood Forming Organs p 126 University of Wisconsin Press Madison 1939
5 Gall E A *Minnesota Med* 38 674 705 (1955)
6 Klemperer P *Bull N Y Acad Med* 30 526 (1954)
7 Watkins C H and Hall B E *Am J Clin Pathol* 10 387 (1940)
8 Wiseman B K in George R Minot Symposium of Hematology (W Dameshek and F H L Taylor eds ) p 554 Grune and Stratton New York 1949

# 12

# The Malignant Lymphomas

LAWRENCE BERMAN

Wayne University College of Medicine Detroit Michigan

The relationship between leukemia and the group of tumors known to American pathologists as the malignant lymphomas is still not clearly defined In the absence of knowledge of the etiologic agents or mechanisms only the most rudimentary classifications of these idiopathic conditions are justified A discussion based on morphologic observations has little hope of reconciling our divergent views into a unified concept Nevertheless it is of value to consider the situation from a historical point of view in order to explain some attitudes shared by a fairly large segment of American pathologists and hematologists [1]

About twenty years after Virchow s discovery of leukemia Cohnheim began the separation of a group of related conditions without the typical findings of leukemia His designation of these as "pseudoleukemia" clearly applies to what we would now recognize as aleukemic lymphadenosis or aleukemic leukemia For a long time pseudoleukemia included a group of diseases of lymphoid tissues characterized by tumefaction of lymph nodes The concept embraced conditions referred to by current authors as lymphosarcoma lymphogranuloma or Hodgkin s disease aleukemic lymphadenosis or aleukemic lymphocytic leukemia as well as various types of reticuloendothelial cell hyperplasias As a result of the ever expanding list of conditions designated as pseudoleukemia the term lost specificity and meaning and it is now obsolete

In 1893 Kundrat described lymphosarcoma as a primary malignant disease of the lymphatic system beginning locally and progressing by direct invasion and lymphogenous metastasis Kundrat s separation of

[1] An excellent review of this subject is available in Watson s chapter in the Handbook of Hematology (11) Much of this historical discussion is based on the literature represented in Watson s extensive bibliography

lymphosarcoma depended on gross anatomic findings It was soon realized that the histologic patterns seen in lesions satisfying Kundrat's concept are readily duplicated by leukemia The gross anatomic features of Kundrat's lymphosarcoma were the reflections of various kinds of cytologic and histologic changes It can be appreciated why the original concept of lymphosarcoma as a special entity suffered the fate of Cohnheim's pseudoleukemia At the time that Kundrat presented his view of the tumorlike or sarcomatous nature of lymphosarcoma the lesions which were considered to be true tumors of lymphoid tissues were not clearly differentiated from benign hyperplasias of these tissues Because of their apparent local and tumorlike origin Kundrat considered certain lesions to be true tumors therefore the term lympho*sarcoma* This was thought to be different from leukemia because leukemic changes appeared to be of multicentric origin and diffuse in form

Most of us today believe that both Kundrat's lymphosarcoma and leukemia are malignant neoplastic diseases but we are faced with an old question Is lymphosarcoma the tumorlike lesion which seems to originate from a single focus and then spread or metastasize a different disease from leukemia which may present similar cytologic features but which usually originates multicentrically and presents lesions which are usually diffuse in character? The answer depends on discovery of etiologies Many of us have seen patients in whom there is an initial lesion involving one site such as the stomach bowel or spleen and in whom the original lesion remains localized for a period followed by evidence of spread to regional lymph nodes as in the manner of carcinomas after which the disease becomes widely disseminated so that ultimately some of the proliferating cells of the lesions appear in the blood as in leukemia An example of this sequence of events was described recently by Dameshek and Bloom (3) The patient a 56 year old woman under continuous observation for a period of 8 years was first seen in 1947 Hepatosplenomegaly and pancytopenia were present without evidence of leukemia By 1950 splenomegaly had become massive and splenic puncture resulted in a diagnosis of lymphosarcoma In 1954 the bone marrow had become infiltrated with lymphocytes thereafter there was an increasing number of atypical lymphocytes in the peripheral blood Recently we have seen material from the stomach of a patient satisfying the original criteria of Kundrat for lymphosarcoma The stomach was involved with a cellular neoplasm composed of lymphoblastic elements There is no other known involvement except for a small lesion of localized nature in one of the gastric nodes removed with the stomach This small area composed of lymphoid cells similar to those in the stomach

tumor might be considered a metastasis by those supporting the concept held by Kundrat.

Clinical observations of a definite sequence from solitary tumor to dissemination and ultimate appearance of leukemia and the similarities of cytological features of leukemias of lymphoid tissue origin and Kundrat's lymphosarcoma indicate that the original term lymphosarcoma has no meaning with reference to histologic structure. Lymphosarcoma is a clinical rather than a histological concept. Unfortunately the term lymphosarcoma at least as it is being used in many quarters among American pathologists and clinicians has lost its original meaning. Many Americans use the term in the same way Cohnheim's term pseudoleukemia came to be used for a group of conditions characterized by malignant hyperplasia of lymphoid tissue and with the same results. Often when an American author uses Kundrat's term he may be referring to the whole or various parts of the group of malignant lymph node lesions including lymphosarcoma, Hodgkin's disease, lymphatic leukemia or aleukemic leukemia and reticulum cell sarcoma of lymph nodes among others.

Another event of historical significance was Sternberg's presentation of his concept of leukosarcoma in 1903. This was characterized by the presence of tumors with aggressive infiltrative growth and atypical cells different from those usually seen in ordinary leukemias. The lesions were associated with the appearance of atypical cells in the peripheral blood and the lesions were more widespread than was the case in Kundrat's lymphosarcoma. The aggressive character of the atypical cells in leukosarcoma and the sudden appearance of such cells in the blood were reasons for separating leukosarcoma from leukemia. In 1938 Richter (9) pointed out several reasons for considering Sternberg's leukosarcoma to be closely related to leukemia. He noted that in cases satisfying Sternberg's criteria there were often changes like those of leukemia in various organs. Furthermore Richter and MacDowell (cited by Richter 9) had made observations on spontaneously occurring lymphatic leukemias in mice as well as transplantation experiments with such material. They showed that all varieties of lesions may occur, thus there appeared to be a fundamental unity of leukemia and leukosarcoma.

Sternberg's early work in 1898 as well as that of Reed in 1902 served to delineate Hodgkin's lesions as a special entity because of the common appearance of the peculiar giant cells of that disease. The so called Sternberg Reed cells of Hodgkin's tissue are considered by many to represent examples of atypical proliferation of pathological reticulum cells (1 8). Since Hodgkin's lesions include such giant cells in addition

to elements of lymphocytoid character and because the anatomic and clinical manifestations of Hodgkin s disease are in many ways similar to those of the conditions already discussed it is not surprising that they are considered to be related in some way The histologic interrelationships among lesions of the malignant lymphoma group have been emphasized by Custer and Bernhard (2)

Analysis of the lymphoma group has been attempted on clinical grounds by those interested chiefly in therapy In general the clinician is interested in a relatively simple classification and the various lesions are often referred to by that abused term lymphosarcoma which might or might not include the various forms of Hodgkin s disease Israels (5) attempted a clinicopathologic classification which included a consideration of histologic structure in addition to such items as the presence or absence of enlarged peripheral nodes hypersplenism leukemia refractory anemia bone disease and skin lesions There is a strong tendency in the United States to regroup the various proliferative and apparently malignant conditions of lymphoid tissue into a single category called lymphoblastoma or malignant lymphoma The basis for this seems to *be the idea that they are all malignant lesions of lymphoid tissues and* that they have their origin from the hematopoietic elements of such tissue The so called reticulum cells which according to anatomists are morphologically very similar to embryonic mesenchymal cells and which form the cellular ground substance of the lymphoid tissues have in addition to other mesenchymal potentialities the capacity of differentiating hematopoietically Therefore they are included among the hematopoietic elements of lymphoid tissue The distinction between the types of malignant lymphomas is based largely on identification of the main cytologic types involved in the neoplastic process This must be done with full knowledge that it is based solely on morphologic appearances although the clinical manifestations of each type are protean and often overlapping Furthermore such distinctions must be made without prejudice as to the question of etiologic relationships or lack of *etiological relationships among* them

The lesions Americans refer to as malignant lymphomas are often referred to by Europeans as the reticuloses or lesions of the lymphoreticular system The last term implies that lymphoid tissues are composed of lymphocytic cells and reticulum cells This is a concept in accord with the ideas of anatomists *Europeans and Americans do not* always use the same terms to designate the various cellular components in lymphoid tissue Without attempting to present a case for the use of one kind of terminology in favor of another in Table I there are in

dicated a small sampling of American (1) Swiss (7) and Italian (6) designations

A common American point of view in relation to the diagnosis of malignant lymphomas was presented by the author in 1953 (1) With respect to the histogenesis of malignant lymphomas the significant cytologic elements under normal conditions in the order of maturation or differentiation were listed as follows (1) reticulum cells (2) hematopoietic reticulum cells and reticular lymphocytes ("lymphoblasts" of American pathologists) (3) lymphocytes (Figs 1 and 2) These represent the hematopoietic elements of lymphoid tissues Usually the pre

TABLE I

CELLULAR HEMATOPOIETIC ELEMENTS OF LYMPHOID TISSUE

| American (Berman) | Swiss (Moeschlin) | Italian (Michelazzi) |
|---|---|---|
| Reticulum cells | (Fibroblasts?) | (Histoid cells) |
| Hematopoietic reticulum cells | Lymphoid reticulum cells | Hemohistioblasts |
| Reticular lymphocytes or lymphoblasts" of American pathologists | Lymphoid reticulum cells<br>Lymphatic plasmoblasts? | Lymphoblasts<br>Prolymphocytes |
| Lymphocytes | Lymphocytes | Lymphocytes |

Terms in parentheses are not considered hematopoietic by some authors

dominating cells of the pathologic proliferation are among those listed above Of course in malignancy it can be expected that cells of these types may appear abnormal Recently Di Guglielmo (4) discussed the relation between lymphosarcoma and leukemia On the basis of finding atypical cells of various types in instances he considered to be lymphosarcoma he felt that it can be distinguished in nearly all cases from chronic lymphocytic leukemia in which the cells were not atypical The tendency among Americans is to place the abnormal elements into one or more of the listed categories as atypical representatives of the cell lines This is especially true of pathologists because many of the atypical features seen in imprints or smears are not apparent in ordinary sections of lymphoid tissues The idea for the grouping of malignant lymphomas was developed in this country mainly by pathologists who have relied on the use of sectioned material The hematologist who uses dry films is able to discover numerous variants which the pathologist is likely to ignore because of the more limited cellular patterns seen in sections

The classification of malignant lymphomas presented by Berman (1) is a convenient working arrangement which can be applied to sectioned material (Table II) When necessary the simple list can be expanded to

TABLE II

CLASSIFICATION OF MALIGNANT LYMPHOMAS

I Reticulum cell type
II *Lymphoblastic type*
III Lymphocytic type
IV Mixed type
- A Hodgkin type
  - 1 Paragranuloma
  - 2 Granuloma
  - 3 Sarcoma*
- B Follicular type
  - 1 *Pre blastomoid*
  - 2 Progression to diffuse form of malignant lymphoma

* Morphologically indistinguishable from the reticulum cell type

include the more precise information gained by inspection of unusual patterns encountered in dry films It is apparent that the classification is based on the predominance of the three main types of cells in the neo plastic tissue Although there are some instances in which the lesion will present features of more than one type of malignant lymphoma they

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PLATE I

From a plate published by Berman (1) with the kind permission of the author and publisher

Fig 1 Imprint normal lymph node The large cell (*r*) is a reticulum cell The cell marked *ly* is a lymphoblast and the cells marked *l* are differentiated small lymphocytes (1050 ×)

Fig 2 Section from the lymph node shown in Fig 1 The cell *r* is a reticulum cell *ly* is a lymphoblast and the cells marked *l* are small differentiated lymphocytes (1050 ×)

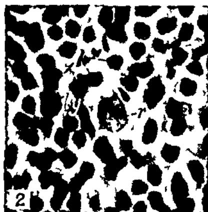
Fig 3 Imprint lymphoblastoma reticulum cell type The large pathological reticulum cells (*r*) have large nucleoli (1050 ×)

Fig 4 Section from the lesion shown in Fig 3 showing reticulum cells (*r*) with nuclei of various sizes (1050 ×)

Fig 5 A giant polyploid reticulum cell in an imprint of a *reticulum cell tumor* (1050 ×)

Fig 6 Imprint lymphoblastoma lymphoblastic type showing lymphoblasts (*ly*) with nucleoli and differential lymphocytes (*l*) (1050 ×)

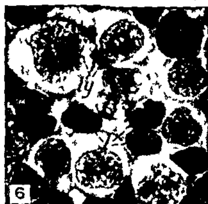

ly
1


ly
2


3


4


5


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form a relatively small percentage of cases It is to be noted that the malignant lymphomas do not include a lesion affecting lymph nodes which is seen in patients with acute leukemia It is important to recognize the fact that the stem cells or lymphoblasts of acute lymphocytic leukemia are quite different from the "lymphoblasts of normal lymphoid tissues The hematopoietic and reticular lymphocytes which represent intermediate forms of lymphocytic cells of normal lymph nodes and which predominate in the lymphoblastic type of malignant lymphoma are often difficult to differentiate from the lymphoblasts of acute leukemia in sectioned material except for the fact that in leukemia the stem cells usually have very little cytoplasm It is unfortunate that the peculiar and special cell seen in acute leukemia and properly known as lymphoblast is confused with the "lymphoblasts seen in malignant lymphomas or normal lymphoid tissue This is largely the result of the appropriation by pathologists of the term lymphoblast to describe the lymphoid hematopoietic reticulum cells and reticular lymphocytes of lymphoid tissues A discussion of the essential differences between the true lymphoblasts of acute lymphocytic leukemia and the relatively undifferentiated lymphocytes of lymphoid tissues is given by Sundberg (10) The morphologic appearances of the lesions in malignant lymphomas as seen in sections and imprints is shown in Figs 3–9 and 17–24 For diagnosis the combined use of sections and imprints is advantageous and often necessary The histologic architecture of the lesions offers indications of their benign or malignant nature whereas the imprints are frequently needed for accurate identification of cell types Usually unless atypical and bizarre cells are present in large number

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PLATE II

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Fig 7 Section from the lymph node shown in Fig 6 The large cell near the center of the field is a reticulum cell The other cells are lymphoblasts (1050 ×)

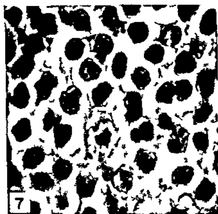
Fig 8 Imprint lymphoblastoma lymphocytic type (1050 ×)

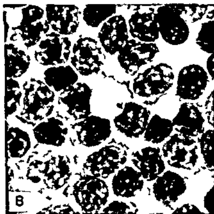
Fig 9 Section from the lesion shown in Fig 8 A few lymphoblasts (*ly*) are present (1050 ×)

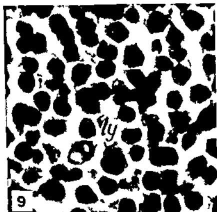
Fig 10 Section of bone marrow of a patient with lymphocytic lymphoblastoma The myeloid tissue is infiltrated with differentiated lymphocytes (1050 ×)

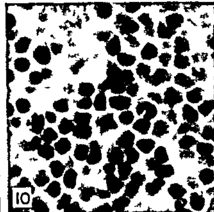
Fig 11 Undifferentiated hematopoietic reticulum cell in a blood smear from a patient with leukemic reticuloendotheliosis (1050 ×)

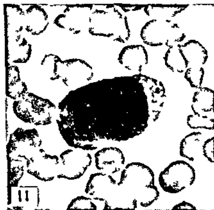
Fig 12 Undifferentiated reticulum cell in a blood smear from a patient with leukemic reticuloendotheliosis (1050 ×)

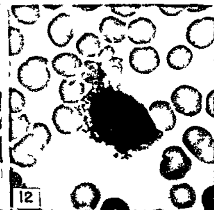

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imprints are not adequate for determining whether or not a lesion is malignant because the elements seen in imprints are also present in normal or reactive lymphoid tissue

The relationships between the malignant lymphomas and leukemias were discussed in detail in a previous paper (1) It is commonly observed that the patient who has a lymph node tumor which the pathologist diagnoses as malignant lymphoma lymphocytic type is an individual who has *peripheral blood manifestations of chronic lymphocytic* leukemia or in whom there are diffuse lesions typical of that disease in various organs and tissues (Fig 10) The diagnosis made depends on how much and what part of the patient is examined In the author's material the lymphoblastic lymphomas are accompanied by a leukemic blood picture in 50% of cases during the course of the disease The frequency with which other authors have encountered a leukemic picture seems to be related to the frequency of blood studies Sometimes a patient with lymphoblastic lymphoma had a disease which began as enlargement of lymph nodes without a leukemic blood picture thus conforming to the original concept of lymphosarcoma Later as the condition became more widely disseminated atypical lymphoid cells appeared in the blood in increasing number so that eventually leukemia was present

Although the reticulum cell type of malignant lymphoma is nearly always aleukemic there are cases in which the peripheral blood contains

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PLATE III

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Fig 13 Undifferentiated cell in a bone marrow smear from a patient with leukemic reticuloendotheliosis The two small cells are differentiated lymphocytes (1050 ×)

Fig 14 Pathologic reticulum cells (*r*) in a tumor of the liver from the patient whose bone marrow is shown in Fig 13 (sectioned material) (1050 ×)

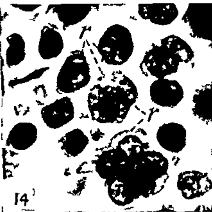
Fig 15 Section of lymph node from a patient with leukemic reticuloendotheliosis The large plurinuclear cell cannot be distinguished from a Sternberg Reed cell Other cells (*u*) are abnormal undifferentiated cells whose nuclei in imprint preparations have the characteristics of reticulum cells shown in Figs 13 and 16 although they resemble lymphoblasts in sections (1050 ×)

Fig 16 Pathologic reticulum cells in a bone marrow smear from a patient with myeloid reticulosis and reticulum cell type of malignant lymphoma (1050 ×)

Fig 17 Section lymph node Hodgkin paragranuloma (585 ×)

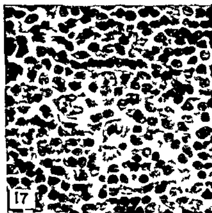
Fig 18 Section lymph node Hodgkin granuloma (585 ×)

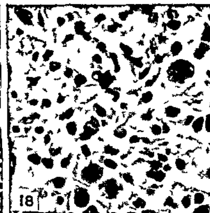

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primitive reticulum cells and their atypical derivatives which are hardly classifiable with existing terminology The free cells originating from the tumors may have a monocytoid or lymphocytoid appearance or they may remain undifferentiated (Figs 11–16) Whereas lymphomas of the reticulum cell type are usually aleukemic and may be regarded as examples of aleukemic reticulosis the instances in which the cells appear in the circulating blood may be regarded as examples of leukemic reticulosis or as is frequently stated leukemic reticuloendotheliosis

In summary the group of lesions classified as the malignant lymphomas by many American pathologists represent partial pictures based on the study of isolated lesions of lymph nodes of patients who may have more generalized and leukemia like conditions The fact that the malignant lymphomas may appear in aleukemic and leukemic forms depending partly on the time of study of the patient and partly on the extent of the study and no doubt partly on the nature of the pathologic process itself indicates that there is a fundamental relationship between the malignant lymphomas and leukemia at least in the way the tissues react to varied and possibly unrelated stimuli Our present ignorance of the nature of these stimuli emphasizes the provisional nature of the entire concept of the malignant lymphomas as a disease entity

## REFERENCES


1 Berman L *Blood* 8 195 (1953)
2 Custer R P and Bernhard W G *Am J Med Sci* 216 625 (1948)
3 Dameshek W and Bloom M *Bull Tufts New Engl Med Center* 1 201 (1955)
4 Di Guglielmo G *Sci Med Ital* (Fr ed) 3 614 (1955)


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PLATE IV

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Fig 19 Section lymph node Hodgkin granuloma (585 ×)

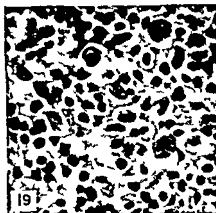
Fig 20 Section lymph node Hodgkin sarcoma The lesion is practically identical with the reticulum cell type of tumor shown in Fig 4 (1050 ×)

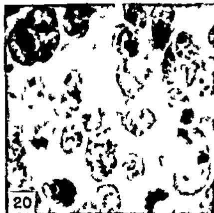
Fig 21 Sternberg Reed cell in an imprint of a lymph node (Hodgkin granuloma) (1050 ×)

Fig 22 Section of the lymph node shown in Fig 21 A Sternberg Reed cell is in the center of the field (1050 ×)

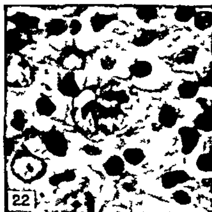
Fig 23 Section of lymph node lymphoblastoma follicular type This lesion contains discrete nodules and is sometimes called a pre blastomoid stage (105 ×)

Fig 24 Section of lymph node lymphoblastoma follicular type showing progression to diffuse type of malignant lymphoma (105 ×)


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5 Israels M C G *Lancet* 525 (Sept 12 1953)
6 Michelazzi A M and De Renzi S in Il Puntato Linfoghiandolare" Nistri Lischi Editori Pisa 1940
7 Moeschlin S *Folia Haematol* **65** 181 (1941)
8 Rebuck J W *J Lab Clin Med* **32** 660 (1947)
9 Richter M N in Handbook of Hematology (H Downey ed) Vol 4 p 2883 Paul B Hoeber New York 1938
10 Sundberg R D *J Lab Clin Med* **32** 777 (1947)
11 Watson C J in Handbook of Hematology (H Downey ed) Vol 4 p 3049 Paul B Hoeber New York 1938

Part V

# Leukocytic Physiology

Chairman
M M Wintrobe
College of Medicine
University of Utah
Salt Lake City Utah

# 13

# Cultivation of Leukemic Cells in the Cartesian Diver

JØRGEN KIELER
Fibiger Laboratories Copenhagen Denmark

The cultivation of leukemic cells *in vitro* has been attempted by numerous authors during the last forty three years With a few exceptions the efforts to establish continuous growth of malignant blood cells have been unsuccessful The mitotic activity frequently stops soon after explantation In some cases cell multiplication continue for a short period at a moderate rate which however is unable to compensate for cell death

Among the exceptions from this general rule are seven mouse leukemias which have been kept *in vitro* in an active state of proliferation for long periods of time The presence of fibroblasts in these cultures has been shown to be essential for the growth of the leukemic cells The literature on this subject has recently been reviewed (2)

The mechanism of the growth supporting effect of the fibroblasts is so far unknown It may be that the mechanical support plays a role but it is also possible that the fibroblasts provide the leukemic cells with various growth promoting metabolites The removal by the fibroblasts of growth inhibiting agents is a third possibility which should be considered

The influence of fibroblasts on the growth of human leukemic cells *in vitro* has been studied repeatedly but satisfactory results have not yet been obtained Until recently progress in the attempts to grow human leukemic cells in tissue culture has been restricted mainly to prolongation of survival time Owing to the contributions by Osgood *et al* (5 6) however this situation has lately changed These authors have developed a method of tissue culture based on gradient principles which have permitted continuous growth and multiplication of hemic cells for up to two years The results obtained by this method indicate

that cell multiplication may depend on oxygen tension among other factors Therefore as pointed out by Osgood it would be desirable to repeat metabolic studies under ideal oxygen tension Such studies have been carried out during the last few months at the cytochemical division of the Carlsberg Laboratory in collaboration with the Fibiger Laboratory

## Material and Methods

Blood samples from normal persons and from patients with leukemia were placed in an incubator at 37 for a few hours Citrate or oxalate was used as anticoagulant After spontaneous sedimentation of the red cells the leukocyte containing supernatant was placed on top of a 30% bovine albumin solution in a centrifuge tube After centrifugation at 1500 r p m for 10 minutes an erythrocyte free suspension of white cells in their own serum could be aspirated from the tube The suspension was diluted with equal parts of Tyrode s solution and samples were removed for the metabolic study

Cell respiration was measured by the Cartesian diver method the principles and technique of which were developed by Linderstrøm Lang (3) and Holter (1) A schematic drawing of measuring apparatus and diver is presented in Fig 1 Divers with a total volume of 30 to 50 $\mu l$ were used The inside diameter of the neck was 1 18 mm The filling of the diver was done at 37 Five microliters of cell suspension with a total number of 5000 to 100 000 cells was placed at the bottom of the diver The diver neck was then closed with three neck seals The first seal consisted of 1 5 $\mu l$ of 0 15 $N$ NaOH This seal absorbed the CO pro duced during respiration The second seal was made of 1 $\mu l$ of paraffin oil which prevented the exchange of water between the interior of the diver and the flotation medium The last seal placed at the mouth of the neck had the composition of the medium surrounding the diver It formed the main obstruction to loss of gas through diffusion into the flotation medium Furthermore by suitable choice of its volume it became the means of adjusting the initial equilibrium pressure of the diver to the desired value In long term experiments with an initial oxygen concentration different from that of room air a cylindrical hollow glass stopper was inserted into the mouth seal the diameter of the stopper being a little less than the inside diameter of the diver neck The specific gravity of the stopper was adjusted in such a way that it would just sink in the flotation medium The stopper has been shown by Linderstrøm Lang and Holter (4) to be an effective means of reducing gas diffusion

When a gas phase was wanted with controlled initial oxygen concentration the filling of the diver was done under water in a plastic gassing chamber After the introduction of the cell suspension the desired mixture of $O_2$ and $N_2$ was led into the diver through a pipet

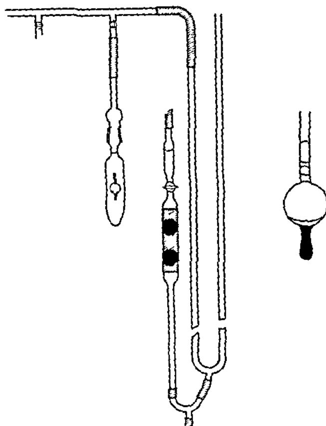


FIG 1 Schematic drawing of diver and measuring apparatus

the tip of which was placed just above the surface of the bottom drop After 20 minutes of flushing the air pipet was removed and the NaOH seal was inserted The air pipet was then reintroduced into the diver neck so that the tip was just above the NaOH seal, and flushing was continued for 5 minutes This procedure was repeated after the insertion of the oil seal and the mouth seal Finally the diver was transferred to the flotation vessel which as flotation medium contained a solution of

$NaNO_3$ and NaCl with small amounts of Na taurocholate (1) The density of this solution was 1 326

The flotation vessel was located in a thermostat at 37 and connected with a manometer with a coarse and a fine screw by means of which the diver could be brought to float at a certain level controlled with a horizontal microscope As respiration proceeded the pressure in the flotation vessel was lowered to make the diver float On the basis of the manometric readings plotted against time the O consumption could be calculated from the formula developed by Linderstrøm Lang (3)

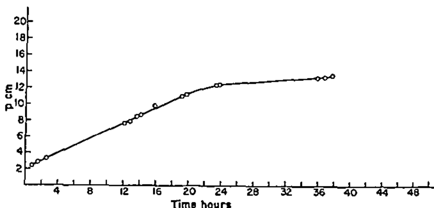



*Fig 2 Respiration of normal cells*

After the experiment the neck seals were removed and the diver was filled with a balanced salt solution A small stirrer was introduced into the diver and after careful stirring samples were removed for cell counting in a Burker Turk counting chamber With the volume of the diver known the total number of cells could be calculated and compared with counts of the cell suspension before the experiment

**Results**

Using the described methods we have followed the respiration of normal and leukemic leucocytes during varying periods of time In the first experiment (Fig 2) the respiration of normal leukocytes was followed for 38 hours The cells were suspended in their own serum diluted with equal amounts of Tyrode s solution The initial gas phase was room air During the first 24 hours considerable oxygen consumption was observed The amount of O consumed per cell per hour was $6\,74 \times 10^{6}$ μl After 24 hours however a decrease in respiration occurred In order to show whether this could be explained by cell death

or nutritional deficiency the neck seals were aspirated after the removal of the diver from the flotation vessel Thus room air was allowed into the diver After careful cleaning of the diver neck new seals were inserted, and the experiment was repeated. It was found that the graph obtained during the second experiment was almost identical with the one from the first experiment Thus the inhibition of respiration after 24 hours of

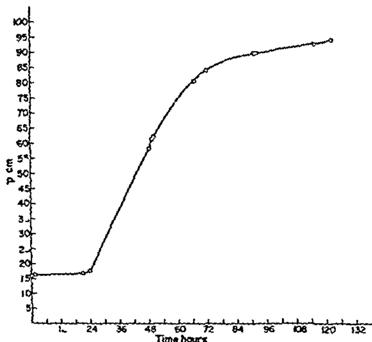



FIG 3 Respiration of leukemic cells patient No 1 acute leukemia

incubation seems to be related to changes in the gas phase Since no changes of pH of the bottom drop could be detected and since pH of the NaOH seal remained above 10 the decrease of respiration could hardly be explained by an accumulation of CO In agreement with this it was found that the inhibition could not be prevented by the use of 0 5 N NaOH

Different graphs were obtained when the diver was charged with leukemic cells (Figs 3 4 and 5) During the first 6 to 24 hours these cells showed a very low rate of respiration This initial phase was followed by a period of intensive respiration lasting 1 to 2 days however

$NaNO_3$ and NaCl with small amounts of Na taurocholate (1) The density of this solution was 1 326

The flotation vessel was located in a thermostat at 37 and connected with a manometer with a coarse and a fine screw by means of which the diver could be brought to float at a certain level controlled with a horizontal microscope As respiration proceeded the pressure in the flotation vessel was lowered to make the diver float On the basis of the manometric readings plotted against time the O consumption could be calculated from the formula developed by Linderstrøm Lang (3)

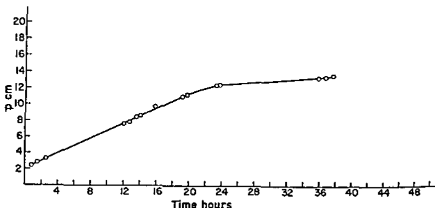



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the beginning of the experiment When respiration had fallen off after 46 hours 13% of the oxygen had been consumed

*In order to obtain further evidence in favor of the assumed influence* of oxygen tension on cell respiration a comparative study of respiration at different initial oxygen tensions was carried out The results of such a study with cells from patient No 3 are shown in Figs 6–10

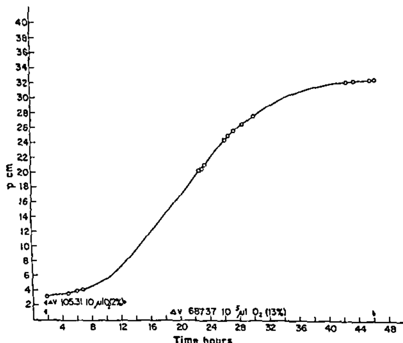



Fig 5 Respiration of leukemic cells at room air patient No 3 chronic lymphatic leukemia

When the gas phase at the beginning of the experiment contained 5% O and 95% $N_2$ only moderate respiration took place The oxygen consumption per cell per hour was 0 68 × $10^{6}$ μl (Fig 6) When the initial oxygen concentration was 10% however respiration increased by a factor of 10 the oxygen consumption per cell per hour being 6 30 × $10^{6}$ μl (Fig 7) If the oxygen concentration was further increased a depression of respiration was observed At 15% oxygen the oxygen consumption was 1 41 × $10^{6}$ μl per cell per hour (Fig 8) At room air it

after which oxygen consumption decreased again Renewal of the gas phase with room air had the same effect as in the case of normal cells—i e respiration started again Changes of pH or saturation of the NaOH seal could not be demonstrated Thus the respiratory inhibition at the end of the experiments can hardly be explained by cell death nutritional deficiency or CO intoxication

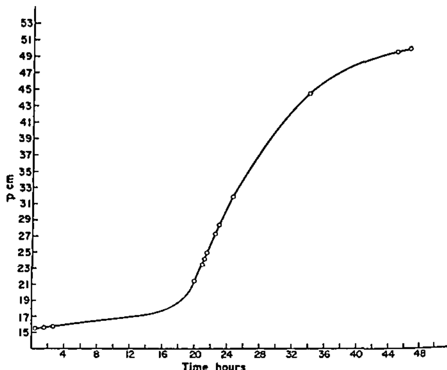



FIG 4 Respiration of leukemic cells patient No 2 chronic myeloid leukemia

If the results of these experiments are compared with Osgood's observations variation in oxygen tension presents itself as a possible explanation of the S shape of the respiration curve of leukemic cells It is conceivable that respiration may be inhibited by high oxygen tensions as well as low oxygen tensions If this alone should explain the shape of the respiration curve of leukemic cells the respiratory system of the latter must be extremely sensitive to variations in oxygen tension In patient No 3 (Fig 5) maximal rate of respiration occurred after about 12 hours of incubation at which time the cells had consumed a total of 105 31 × $10^3$ μl of O or 2% of the oxygen present in the diver at

the beginning of the experiment When respiration had fallen off after 46 hours 13% of the oxygen had been consumed

In order to obtain further evidence in favor of the assumed influence of oxygen tension on cell respiration a comparative study of respiration at different initial oxygen tensions was carried out The results of such a study with cells from patient No 3 are shown in Figs 6–10

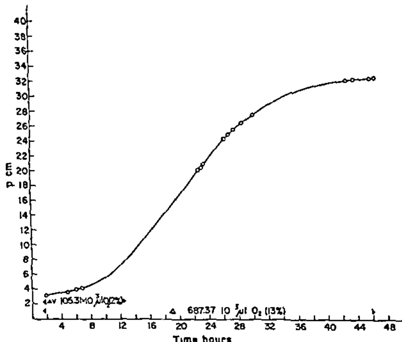



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was $0.40 \times 10^{-6}$ µl (Fig 9) and at 30% oxygen only $0.21 \times 10^{-6}$ µl (Fig 10)

Similar results were obtained with other leukemic cells but not with normal leukocytes In Table I the results obtained so far are sum

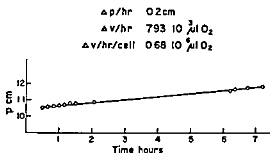



Fig 6 Respiration of leukemic cells at 5% O + 95% N patient No 3 chronic lymphatic leukemia

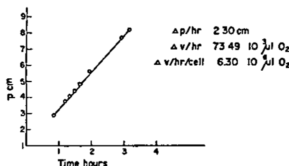



Fig 7 Respiration of leukemic cells at 10% O + 90% N patient No 3 chronic lymphatic leukemia

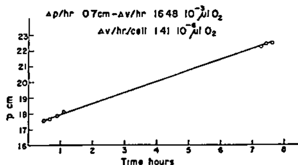



Fig 8 Respiration of leukemic cells at 15% O + 85% N patient No 3 chronic lymphatic leukemia

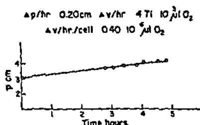



Fig 9 Respiration of leukemic cells at room air patient No 3 chronic lymphatic leukemia

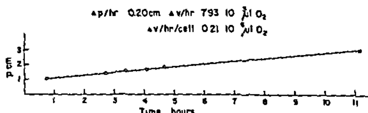



Fig 10 Respiration of leukemic cells at 30% O + 70% N patient No 3 chronic lymphatic leukemia

marized It appears from the table that the O consumption per normal leukocyte per hour is about ten times as high as that of leukemic cells at room air Furthermore it is seen that the respiration of normal leukocytes *is independent of variations in oxygen concentration* In all three types of leukemia so far investigated however cell respiration was found to be influenced by oxygen tension Maximal respiration was found at

TABLE I

O Consumption Per Cell Per Hour at Various O Tensions

(μl × 10⁻⁶)

| Diagnosis | Case | Per cent $O_2$ 5 | 10 | 15 | Room air | 30 |
|---|---|---|---|---|---|---|
| Acute stem cell leukemia | 1 | 0 17 | 0 76 | | 0 50 | |
| | 2 | | | | 0 37 | |
| | 5 | | | | 0 50 | |
| Chronic myeloid leukemia | 4 | 0 00 | 5 94 | | 0 76 | |
| | 6 | | | | 0 60 | |
| Chronic lymphatic leukemia | 3 | 0 68 | 6 30 | 1 41 | 0 40 | 0 21 |
| Normal | | 6 10 | 6 93 | | 6 74 | 6 33 |

was 0 40 $\times$ 10[6] $\mu$l (Fig 9) and at 30% oxygen only 0 21 $\times$ 10[6] $\mu$l (Fig 10)

Similar results were obtained with other leukemic cells but not with normal leukocytes In Table I the results obtained so far are sum

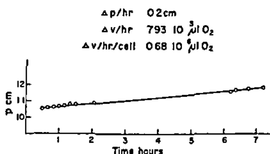



Fig 6 Respiration of leukemic cells at 5% O + 95% N patient No 3 chronic lymphatic leukemia

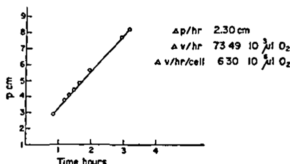



Fig 7 Respiration of leukemic cells at 10% O + 90% N patient No 3 chronic lymphatic leukemia

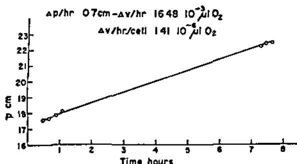



Fig 8 Respiration of leukemic cells at 15% O + 85% N patient No 3 chronic lymphatic leukemia

however When after the initial phase of moderate respiration in patient No 3 (Fig 5) the increase of respiration occurred only 2% of the total oxygen had been consumed Thus the oxygen concentration at this time was 19 to 20% When respiration was at its maximum after 20 to 24 hours the oxygen concentration in the diver was 18% The O consumption per cell per hour at this time was 2.41 × $10^{-6}$ μl as compared to 6.30 × $10^{6}$ μl at 10% oxygen 1.41 × $10^{6}$ μl at 15% oxygen and 0.40 × $10^{6}$ μl at room air (see Table I) This seems to indicate that too low values have been obtained in the short term experiments owing to the presence of an inhibiting factor which disappears in the course of the first 24 hours Future research will have to clarify the nature of this factor Finally when the respiration curve in patient No 3 leveled off after 46 hours of incubation the oxygen concentration was still 17% after a consumption of 13% of the initial amount of oxygen It is likely that the same factors are responsible for the shape of the terminal part of the respiration curve of normal as well as of leukemic cells

Although the experimental conditions under which the cells are kept in the Cartesian diver undoubtedly have a deciding influence on the shape of the respiration curve the influence of oxygen tension on respiration of leukemic cells is probably a more general phenomenon The mechanism of this effect remains to be clarified It is in all probability of enzymatic nature but it is not necessarily a direct effect on the respiratory enzymes The action of the cytochrome system is known to be only slightly sensitive to variations in oxygen tension The absence of these enzymes in leukemic cells may therefore be expected A study of the cytochromes in leukemic cells has been commenced in collaboration with Dr K M Møller of the Carlsberg Laboratory The preliminary results indicate that cytochromes are present in leukemic cells at least at small concentrations This observation suggests that the oxygen sensitivity is due to the presence of an inhibitor rather than to the absence of the enzymes found in fully differentiated cells

The sensitivity to variations in oxygen tension may be characteristic of all immature cells It offers an explanation of the fact that mitotic figures are extremely rare in the circulating blood even in cases with numerous mitoses in the bone marrow A parallel phenomenon is observed in some ascites tumors where mitotic figures are seen only in the peritoneum but not in cells suspended in the ascitic fluid Furthermore the effect of fibroblasts on the growth of mouse leukemia *in vitro* should be evaluated with the oxygen sensitivity in mind This effect may be due to a reduction of the oxygen tension in the immediate neighborhood of the leukemic cells In support of this assumption it can be men

10% oxygen At this concentration cellular respiration reached normal values in the case of chronic myeloid leukemia and chronic lymphatic leukemia The lower value obtained with cells from a case of acute stem cell leukemia may be explained by the fact that these cells had been grown in suspension in roller tubes for 10 days before their sensitivity to variations in O tension was studied This assumption is in agreement with observations made with a mouse carcinoma which showed a constant rate of respiration during one month when the cells were kept in an active state of proliferation in a clot in roller tubes When the cells were grown in suspension however cell multiplication stopped and respiration decreased at the same time After 2 weeks of incubation respiration had decreased by a factor of 10 In patient No 1 the oxygen consumption per cell per hour at room air was 0 50 $\times 10^{-6}$ µl when measured shortly after the blood aspiration from the patient After 10 days of incubation however the oxygen consumption was 0 07 $\times 10^{-6}$ µl per cell per hour

**Discussion**

From the experiments described above we can conclude that in contrast to normal leukocytes the respiration of leukemic leukocytes is dependent on the oxygen tension The optimal tension was obtained by decreasing the oxygen concentration by 50% as compared with that of room air At the optimal tension the respiration of leukemic cells is of the same order as that of normal cells but it decreases considerably at higher or lower oxygen tensions

Furthermore this study has shown that the shape of the respiration curve in long term experiments cannot be explained by variations in O tension alone Evidently the decrease in respiration of normal cells on the second day cannot be explained by a decrease in oxygen tension since this would not have any influence on the oxygen consumption by normal leukocytes As previously mentioned no accumulation of CO took place On the other hand a decrease of CO tension below a critical level may play a role This possibility will have to be studied by other methods than the Cartesian diver technique in which the complete absorption of CO by the NaOH seal is essential for the correct measurement of oxygen consumption The absence of pH changes in the bottom drop does not support the last mentioned hypothesis The colorimetric method used for pH determinations in these experiments may not have been sensitive enough however

Variations in oxygen tension have probably influenced the shape of the respiration curve of leukemic cells Other factors must be at work too

# 14

# Observations on Human Leukemic Cells in Culture[1]

EDWIN E. OSGOOD

Division of Experimental Medicine University of Oregon
Medical School Portland Oregon

The development of three methods of tissue culture based on gradient principles has made possible the long term culture of human leukemic cells isolated from blood (13 15) Because of limitations of time and space the observations recorded in these three references will not be repeated and it will be assumed that the reader has these papers available The observations reported in this paper have been made in collaboration with Mr John H Brooke and Mr James McNeese cytologists in the Division of Experimental Medicine who have done the culture work and most of the photography

All strains of hemic cells with the exception of the plasmocytic and thrombocytic series have now been isolated from blood and grown for periods long enough to exceed the life span of the differentiating cells which makes it seem improbable that a fixed tissue cell is necessary for the continued growth of any hemic series of cells At the time of writing however only the two strains of the monocytic series J 96 and J 111 are growing well enough for general distribution although strains of the granulocytic series isolated from chronic granulocytic leukemic blood J 113 J 115 J 118 and J 128 have been cultured for as long as 297 60 106 and 175+ days respectively when all but J 128 were lost due to a series of laboratory accidents such as contamination or defective thermostats J 128 is growing well at over 175 days and barring accident should be available for distribution within the next year These accidents have emphasized the importance of keeping replicate cultures in separate

[1] Aided in part by grant C-2350 from the National Institutes of Health U S Public Health Service and Contract AT(45-1) 581 from the U S Atomic Energy Commission

tioned that the growth promoting effect is obtained only if the inter space between leukemic cell and fibroblast is very small Similarly the terminal stage of agranulocytosis seen in many cases of leukemia may be explained by the ousting of normal reticulum cells in the ab sence of which respiration of the leukemic cells decreases and cell multiplication stops

Finally it should be pointed out that the present results are in agree ment with Osgoods pioneer work Further progress in the attempts to cultivate leukemic cells *in vitro* may be obtained by the introduction of a controlled gas phase

## Acknowledgments

This study was carried out at the Cytochemical Division of the Carls berg Laboratory in collaboration with the Fibiger Laboratory The author wishes to thank Dr and Mrs Heinz Holter as well as Dr K Max Møller for their kind interest helpful advice and generous assistance Acknowledgment is made of the support received from the Lady Tata Memorial Foundation the Anders Hasselbalch Anti Leukemia Fund King Christian Xs Memorial Fund and Holms Fund

## REFERENCES

1 Holter H *Compt rend trav lab Carlsberg Ser chim* **24** 399 (1943)
2 Kieler J *Rev hematol* **10** 516 (1955)
3 Linderstrøm Lang K *Compt rend trav lab Carlsberg Sér chim* **24** 333 (1943)
4 Linderstrøm Lang, K and Holter H *Compt rend trav lab Carlsberg Ser chim* **24** 105 (1942)
5 Osgood E E and Brooke J H *Blood* **10** 1010 (1955)
6 Osgood E E and Krippaehne M L *Exptl Cell Research* **9** 116 (1955)

or without a long narrow cover slip inserted in the tube on which the cells may settle This method has proved satisfactory for initial isolation of cells for it provides a gradient It does not, however permit calculation of the ideal gradient factor

These two monocytic strains will now grow on almost any medium that is commonly used in tissue culture but we have been unable to determine any advantage of adding embryo extract to the media Excellent growth is obtained on 20% horse serum 20% horse serum ultrafiltrate and 60% acetate balanced salt solution (15) Perhaps the simplest media on which they grow well is either 20% human pleural fluid filtered through a Corning ultrafine sintered glass filter or 20% horse serum with 80% acetate balanced salt solution (15) Dr Robert N Hull in charge of the tissue culture research Biological Research Division of Eli Lilly and Company Indianapolis reports that the strain we sent him of J 96 shows very good growth on a medium of 5% horse serum with 95% Medium TC 199 [3] These cultures grow well on many different balanced salt solutions with 20 to 40% concentrations of human serum human pleural fluid filtered or aseptically collected or horse serum with or without TC 199 Sodium acetate appears to be a desirable ingredient of the medium and essential for successful lymphocytic cultures but Tween 80 is definitely harmful to the lymphocytic series and causes leaks in the slide cap cultures by dissolving the beeswax used to seal the cover slip to the vial lip

Slanted test tube cultures of these monocytic strains have been successfully shipped by air express with a label "Do Not Freeze" as far as Buffalo New York and Houston Texas and cultures have been transported by plane to the National Cancer Institute and by automobile from Portland to Galveston and survived the trip

Both J 96 and J 111 retain their ability to phagocytose finely divided carbon (Plate I Fig 2)

The extreme pleomorphism of the cultures of the monocytic series of cells (Plate I Fig 3) persists to date This pleomorphism is evident 24 to 48 hours after the cells are isolated Awrorow and Timofejewsky (1) in 1914 were the first to demonstrate this The literature on the formation of giant cells from monocytes in culture has been recently reviewed by Milton N Goldstein (6) Some of the cells resemble fibroblasts so closely that one wonders if some of the cultured cells previously called fibroblasts may not actually have been derived from cells of the monocytic series since cells of the monocytic series undoubtedly are widely

[2] Made by cutting No 1 Goldseal 24 × 60-mm cover slips longitudinally in half

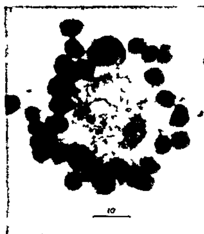
[3] Obtainable from Difco Laboratories Detroit 1 Michigan

incubators not changing media on all cultures of the same strain from the same lot of medium at the same time and distributing the cells as widely as possible as soon as they are growing well

It has been observed that the patterns of growth are so different and develop so early in the gradient culture (see figures in references 14 and 15 and in this paper) that the gradient culture technique can be adapted to identification of the type cell of the acute leukemias and will give a definite answer within 24 to 48 hours The leukemic cell if the gradient factor is properly adjusted outgrows the corresponding normal cell to such an extent that it indicates that there is a differential rate of multiplication in favor of the leukemic cell The detailed evidence for these statements will be presented elsewhere

The role of the gradient factor in controlling conditions necessary for successful cell growth has been confirmed in many different ways For example cultures either from cells over a year in culture or from freshly isolated cells set up by the inverted slide cap technique with replicates of the same cell count but a series of different depths may show optimal growth at one depth and yet show complete disappearance of the cells in shallower and deeper cultures The same phenomenon may be observed if the depth is kept constant and the gradient factor is changed by varying the cell count There are microenvironments on the gradient slide and in other types of cultures (see Plate I Fig 1 and its legend) Similar microenvironments may have much to do with the location of metastases within the body and in determining whether a malignant change in a cell or group of cells leads to the establishment of a malignant tumor or only to a cancer *in situ* which may ultimately regress In other words there is an ecology of cells within the body as well as with in cultures Although the two strains of cells which have been cultured longest were isolated from a so called myelomonocytic or Naegli type J 96 and a typical Schillings type of monocytic leukemia J 111 they show essentially identical characteristics in culture and anything that is said or illustrated about one of these strains could be duplicated in *comparable preparations of the other This suggests that there may be* no real difference between these forms of monocytic leukemia

The longer these strains have been cultured the easier they are to grow J 96 *will have been two years in culture on April 7 1956 and* J 111 on August 30 1956 and they now will grow well under any of the standard techniques of tissue culture One of the simplest techniques of growing them is in a stationary 1 9 × 15 cm test tube in a rack at an angle of 10 to 20 degrees with 10 ml or more of culture medium with


10


10


10



## PLATE I

All figures are from Wright s stains and the magnification is indicated on the figures

Fig 1 Photomicrograph of neutrophil myelocyte surrounded by lymphocytes from a gradient slide of culture J 107 removed on the sixth day after the culture was started from the blood of a patient with chronic lymphocytic leukemia The field was selected from the zone between that for optimal growth of lymphocytes which resembled Fig 4 in reference 15 and the region higher on the slide with lower gradient factor which resembled Figs 7 and 8 in reference 14 to illustrate the presence of microenvironments in addition to the major gradient in these cultures

Fig 2 Photomicrograph to illustrate the ability of even the fibroblast like or reticulum cell like cells to phagocytose carbon Culture J 111 from the blood of a patient with acute leukemic monocytic leukemia of the so called Schilling type 468 days after isolation and 48 hours after addition of finely divided carbon to a slanted test tube culture containing a narrow cover slip on which the cells could settle (cf Plate III Fig 2 which shows a replicate culture on the same day under phase microscopy)

Fig 3 Field from the gradient slide near the upper zone of good growth from culture J 111 made on the eighth day after isolation to illustrate that the extreme pleomorphism occurs very early In this one field are several multinucleated giant cells resembling the Langhan s and foreign body type and also mononuclear giant cells The predominant cells resemble reticulum cells There are some typical promonocytes such as were in the original blood and a few fibroblast like cells These are the first type of cell to appear when cells of the monocytic series are cultured and are the predominant type of cell on this slide at intermediate gradient factors

Fig 4 Higher power photomicrograph from the same general area of the same slide as Fig 3 to show the typical nuclear morphology of the Langhan s type giant cells As many as 48 nuclei have been counted in some of these cells but cells with fewer nuclei were selected for photography because they show the nuclear detail better *Similar cells are found in both J 111 and J 96 cultures after more* than 18 months in culture

usually reported but these phase preparations are unlikely to provide the optimum gradient factor for ideal growth Further studies are certainly indicated with colchicine and with varying times and concentrations of exposure to a calcium binding EDTA solution to see if the time of mitosis in optimally growing cultures can be determined Although accurate chromosome counts have not been possible in these preparations the majority of the cells in mitosis appear to have numbers and sizes of chromosomes approximating the usual human diploid number and size

Phase microscopy reveals features of these cells not well shown in the fixed preparations Not only are these cells variable in morphology shape and size but they also show an extraordinary variety of form and motion of pseudopodia size and number of nucleoli and intracytoplasmic granules inclusions and phagocytized particles (Plate II Figs 3 and 4 Plate III Figs 1–4) These together with the processes of phagocytosis pinocytosis and clasmatosis are shown even better in a time lapse phase moving picture [4] This moving picture also shows a short sequence made at 41 days after isolation of the granulocytic leukemia culture J 118 which was subsequently lost owing to infection and a short sequence of the Maben adenocarcinoma of the bronchus cell after 710 days in culture which was isolated by Dr Arthur Frisch (5) illustrating the differences between monocytic and epithelial cultures and showing that the rotating nuclei appear in both but that the pseudopodia growth pattern and active secretion of mucus in the bronchogenic carcinoma are differentiating features in addition to the morphology and widely different gradient factors (15) In this moving picture oscillations of the mitotic apparatus (9) as a whole in dividing cells from culture J 96 are demonstrated

These monocytic leukemia cultures are now being used by others for numerous diverse studies

We are indebted to Dr Joseph Leighton (8) of the National Cancer Institute for the preparations from which the photomicrographs on Plate IV were made Note that Fig 1 of Plate IV resembles a section from reticulum cell sarcoma but in the center of the marked area are nucleated cells of the erythrocytic series which is further evidence of the importance of the microenvironment because the erythrocytic series requires the highest gradient factor of all cell series so far studied Some might regard this as evidence that the monocytic series can give rise to

[4] Made by John Brooke using the facilities of Dr C M Pomerat s laboratory at the University of Texas at Galveston through the courtesy of Doctor Pomerat and his staff and shown after presentation of this paper

distributed throughout the tissues (12) These and the cells resembling the tissue histiocyte or reticulum cell are the predominant cells in these cultures but cells resembling Langhan s foreign body giant cells and Dorothy Reed cells are very common (Plate I Figs 3 and 4 Plate II Figs 1 and 2 and Figs 3 to 8 in reference 14) Nearly all the types of giant cells so well described and pictured by Rebuck (16) which might be derived from histiocytic or monocytic tissue are seen in these cul tures The particular form the cell takes seems to be a function of the micro environment and of the amount and character of the material available for phagocytosis It is noteworthy that the Langhan s type giant cells are commonest near the surface where there may be floating lipid material and near the bottom of the gradient slide where there may be disintegrating debris rich in lipids When the cells are sparse the fibro-blast like form seems to be the preferred pattern Where cells are thickly aggregated the epithelioid and reticulum cell forms predominate

Whether fusion of cells or endomitosis with polyploidy or polyteny is the mechanism of production of these giant cells remains to be deter mined when we have cultures started from a single cell followed by time lapse photomicrography We have not as yet however observed the fusion of cells which Goldstein (6) mentions but have seen polyploidy (Plate II Fig 1) and polyteny (Plate II Fig 2) We have noted more than once a *single* one of many nuclei within a giant cell in mitosis and also multiple metaphase plates within a single giant cell

Studies of the mitotic process in these cells have shown a wide range of chromosome number and morphology but the most puzzling feature has been the relative paucity of visible mitoses in stained preparations Even in the slide cap preparations (14) where the total number of cells increased by 7 6 times between 25 hours and 120 hours mitoses were scarce The cover slip with colchicine from which Figs 1 and 2 of Plate II were derived was covered as solidly with cells as the slide shown in Fig 3 of Plate I but mitoses were abundant in only a relatively nar row zone near one end of the cover slip Most of the smears from the supernatant of bulk cultures have also shown relatively few mitoses but one bulk culture of J 96 in which calcium EDTA and disodium EDTA had been used to free the cells stuck to the glass showed great numbers of mitoses Whether this means that calcium binding by disodium EDTA can arrest mitoses or whether mitoses occur in waves in these cultures is as yet unknown The most plausible explanation seems to be that mitosis requires much less time under ideal conditions than in the usual type of preparation used for timing mitosis We have timed mitosis in living phase preparations in J 96 cells at 57 minutes which is of the order

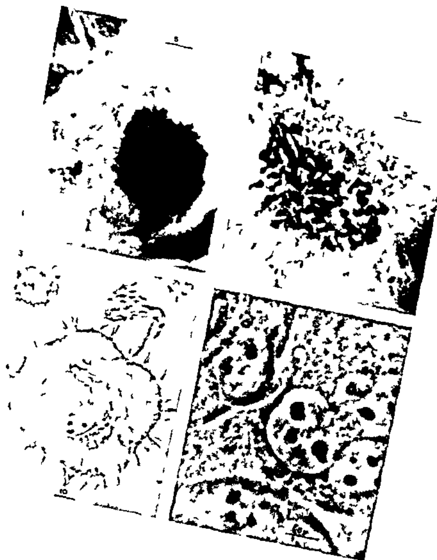


PLATE II

Figures 1 and 2 are Giemsa stains and Figs 3 and 4 are phase photomicrographs

FIG 1 Polyploid mitosis in a giant cell culture J 96 from a cover slip preparation in a slanted test tube 20 hours after addition of sufficient colchicine to make the final concentration 1 300 000 and 555 days after isolation from the blood of a patient with so called Naegli type of acute monocytic leukemia

FIG 2 A probably polytene metaphase of mitosis in a giant cell from the same preparation as Fig 1

FIG 3 Mononucleated giant cell from culture J 96 photographed with phase on day 401 of the culture with sharp focus on the microfibrillae type of pseudopodia which show active wavelike movements in time lapse moving pictures Note also the more rounded pseudopodia and clasmatosis in the smaller cell in the same field which more closely resemble the pseudopodia seen in promonocytes freshly isolated from blood

FIG 4 Phase photomicrograph at high magnification of an area of dense growth similar to those illustrated in Figs 3–6 of reference 14 from culture J 96 after 613 days in culture with sharp focus on the huge nucleoli of cells resembling reticulum cells and Dorothy Reed cells


1
10


2
10


3
10


4
10

PLATE III

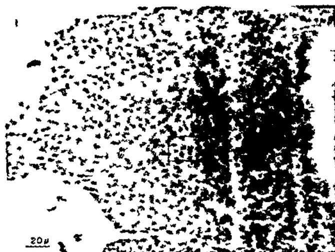
Phase photomicrographs of living cultures from the blood of acute monocytic leukemia in the presence of finely divided carbon to show different phases of the ingestion of the carbon particles and the extreme variation in the form of the pseudopodia even from the same cell

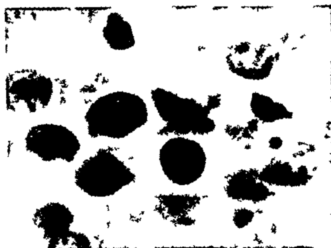
FIG 1 Group of reticulum cell sarcoma like cells growing in close approximation to each other shown at relatively low magnification Most of the granules within the cells are the normal intracytoplasmic granules of the cells but the larger particles and particularly the particles near the end of the longer pseudopod at the top of the picture are recently ingested carbon particles This photomicrograph was made only a few minutes after the carbon was added to the culture but at 610 days after culture J 96 was isolated from the blood

*FIG 2 Culture J 111 468 days after isolation and 48 hours after addition of* carbon to the culture showing two cells in close apposition one of which has accumulated much carbon in the perinuclear area Note in addition to the two long *pseudopodia which extend out of the picture the numerous finer and smaller* pseudopodia along the upper and lower margins of the cells Why two such similar healthy appearing cells in such close apposition have accumulated such widely different amounts of carbon is not easy to explain The great majority of the cells in this preparation had amounts of carbon comparable to that in the lower cell (cf Plate I Fig 2 which is a replicate preparation stained with Wright s stain)

FIG 3 Small group of cells from the same preparation as Figs 1 and 4 in this plate photographed a little longer after the carbon was added than in Fig 1 to show a somewhat later phase in the ingestion of the carbon particles and to illustrate the wide variety of pseudopodia The type of pseudopodia seen projecting toward the bottom of the picture—broad fingerlike processes—are usually the first to be noted after the mount is made Note at the top of the picture the numerous carbon particles along the long slender pseudopodia at various stages of migration toward the center of the cell The fine hairlike pseudopodia to the right of these are similar to the microfibrillae shown on the giant cell in Plate II Fig 3 to the left and right are branched pseudopodia shown better in Fig 4 of this plate At the upper right are more rounded pseudopodia or wavy membranes of the type more commonly associated with the promonocyte of the blood

FIG 4 Another photograph of culture J 96 from the same preparation as Figs 1 and 2 made at 610 days with sharp focus on the branched pseudopodia This reveals the extremely fine branching at the tips of these pseudopodia which was not visible to the naked eye Note also the rounded wavy membrane types of pseudopodia with pinocytosis and the clasmatosis occurring in these same cells All this fine detail is lost in fixed preparations but may be even more dramatically seen in time lapse moving pictures


20μ



## PLATE IV

We are indebted to Dr Joseph Leighton (8) of the National Cancer Institute for permission to use these photomicrographs made by John Brooke in this publication Dr Leighton sent us hematoxylin eosin stained sections which he prepared from a culture of J 96 grown by his cellulose sponge technique The cells had been cultured for a year in our laboratory before he received them

FIG 1 Low power view of a section of a cell clump growing in the interstices of the cellulose sponge Note the resemblance to reticulum cell sarcoma and note particularly the marked area shown at higher magnification in Fig 2 in which *nucleated* cells of the erythrocytic series are seen This is another example of the presence of microenvironments as such nucleated erythrocytes were found only in the center of such areas of dense growth We interpret this as being due to the high gradient factor required by the erythrocytic series

FIG 2 Marked area of Fig 1 shown at higher magnification to demonstrate the typical nuclear morphology of the rubricyte near the center of the field and the pyknotic nucleus of the metarubricyte near the top of the field Note also that there are no intermediate stages between the reticulum cell forms and the nucleated erythrocytic series

culture They adhere to each other and to the glass so firmly that this has prevented the accumulation of some of the types of quantitative data we should like to have The best method we have found so far is the addition of disodium EDTA to give a final concentration of 150 mg per 100 ml 30 minutes before the culture is shaken and sampled This technique gives beautiful morphology and counts but even if the medium is washed from the cells as soon as possible it does impair subsequent growth though not prevent it It also prevents using the preconditioned medium from the culture as part of the new medium for the subcultures We are concentrating our efforts on the problem of culturing other leukemic strains from blood until they are sufficiently adapted to cultural conditions to be easily grown by others We have had several strains of chronic granulocytic leukemia out almost to this stage and then lost them We now have another culture J 128 out at over 175 days which we hope can soon be made available to others We have not been able to separate normal monocytic series from the granulocytic cells however since the gradient factor of the monocytic series completely overlaps that of the granulocytic series Possibly we can accomplish this separation by single cell isolation or by the use of finely divided iron filings The plasmocytic series offers promise of being the easiest of all to grow but we have no cases of plasmocytic leukemia at present The lymphocytic and erythrocytic series offer much more difficult problems because of their high gradient factors It may be that we will have to grow them in combination with J 111 or J 96 in a way comparable to the successful animal lymphocytic cultures obtained by Bichel (2) and de Bruyn (3) We do have definite evidence (Plate IV Fig 2 and similar cells in smears from gradient bulk cultures of J 96 and J 111) that cells of the erythrocytic series have persisted with good morphology and in all stages from the rubriblast to the mature erythrocyte in our cultures for over a year but to date they have always been very scarce relative to the number of cells of the monocytic series in these cultures We have no facilities for electron microscopic studies but Formvar films placed in the bottom of inverted slide cap cultures would enable one to examine these well spread-out cells *in situ* and might reveal much of interest

We have done no work with purely synthetic media and we have not as yet any comparable normal strain of the monocytic series although we have noted monocytic cells growing in every blood culture of each type of leukemia so far studied so that it seems certain that they can be isolated by similar technique from normal blood Attempts to isolate and culture the thrombocytic series have not as yet been made as

cells of the erythrocytic series but we were unable in any of the sections or in any of our other cultures in which we have also found nucleated erythrocytes to see any cells intermediate between the reticulum cell type and the nucleated erythrocytic series and it must be remembered that there were nucleated erythrocytes as well as immature cells of the granulocytic series in the initial blood from which these cells were cultured (14) Only when cultures derived from single identified cells of the monocytic or histiocytic series have been made and studied can this point be settled with certainty Doctor Leighton also reports and has sent us preparations showing good growth on fragmented Gelfoam and he regards this as one of the best methods of maintaining these strains

Dr Harry Eagle (4) at the National Cancer Institute is studying the amino acid requirements of J 111

Dr Arthur Frisch and collaborators (5) of the Bacteriology Department at the University of Oregon Medical School have shown that these monocytic cultures support the growth of the three standard strains of poliomyelitis virus and show typical cytopathogenic effects but will not support the multiplication of influenza or mumps virus Dr Robert N Hull (personal communication) of Eli Lilly and Company is also studying the place of J 111 and J 96 in virus research Dr Milton N Goldstein (6) of the Roswell Park Memorial Institute Buffalo New York is studying giant cell production in cultures of these cells Dr T C Hsu of the M D Anderson Hospital Houston Texas in collaboration with Dr Paul Morehead is studying the mitotic pattern and chromosome morphology of J 111 and J 96 by the hypotonic medium technique (7)

Dr Joseph Burchenal (personal communication) of the Memorial Hospital and Sloan Kettering Institute reports that he has strain J 111 growing well in hamster cheek pouches using cortisone pre treated hamsters as employed by Toolan in her outstanding work (17)

There are still many things to be done with these cultures We have not as yet studied the various antileukemic chemotherapeutic agents We have not determined which of the various conditions (oxygen tension oxidation reduction potential conditioning of medium removal of mature cells) regulated by the gradient factor are the most important but apparently all of them are of some importance We have not been able to define more quantitatively what the ideal oxidation reduction potential or oxygen tension is We have not been able to devise a quantitative definition of when medium should be changed or subcultures made

Our most baffling problem has been to get a uniform suspension of separate cells and still preserve the cells in good condition for sub

7 Cell morphology may be a less effective criterion of cell identification than such features as growth pattern character of pseudopodia and histochemical tests Response to various stimuli under phase contrast time lapse moving pictures might tell us even more about the character of the cells under investigation

8 Great variety of pseudopodia growth patterns and striking phagocytic ability for finely divided carbon particles have been demonstrated in cells presumably of the monocytic series

9 Time lapse moving pictures reveal rotation of nuclei oscillations of the mitotic apparatus pinocytosis and clasmatosis in these same cells

10 It is hoped that the availability of these easily grown hemic cells unencumbered by supporting tissues will prove valuable to others in studies of such widely divergent fields as nutrition and metabolism chemotherapy of malignancies virology bacteriology and mechanisms of immunity and resistance and make possible studies of the contributions of hemic cells to the composition of their environment

## REFERENCES

1 Awrorow P P and Timofejewsky A *Virchows Arch pathol Anat u Physiol* **216** 184 (1914)
2 Bichel J *Arch exptl Zellforsch* **24** 27 (1940-1942)
3 de Bruyn W M *Cancer Research* **9** 593 (1949)
4 Eagle H *Science* **122** 501 (1955)
5 Frisch A W Jentoft V Barger R and Losli E J *Am J Clin Pathol* **25** 1107 (1955)
6 Goldstein M N *Anat Record* **118** 577 (1954)
7 Hsu T C *J Natl Cancer Inst* **14** 905 (1954)
8 Leighton J *J Natl Cancer Inst* **12** 545 (1951)
9 Mazia D *Am Scientist* **44** 1 (1956)
10 Novick A and Szilard L *Science* **112** 715 (1950)
11 Osgood E E in A Symposium on the Blood and Blood Forming Organs" p 219 University of Wisconsin Press Madison 1939
12 Osgood E E *Blood* **9** 1141 (1954)
13 Osgood E E *Ann N Y Acad Sci* **59** 806 (1955)
14 Osgood E E and Brooke J H *Blood* **10** 1010 (1955)
15 Osgood E E and Krippaehne M L *Exptl Cell Research* **9** 116 (1955)
16 Rebuck J W *J Lab Clin Med* **32** 660 (1947)
17 Toolan H W *Cancer Research* **13** 389 (1953)

thrombocytic leukemias are scarce and because the question could always arise if marrow were used of whether the strain cultured might not have been derived from a fixed tissue cell We feel that our early observation (11) that cells of the thrombocytic series appear to grow better in plasma than in serum may be of help in such cultures when suitable material becomes available

It is easy to devise enough investigations using these cells to keep many persons busy for a lifetime and we are always glad to aid anyone in getting started on work with these cell strains—J 111 and J 96 now available for distribution and other strains as they become available The initial isolation of such strains is still far from simple as many cells disintegrate before the gradient factor has become established and the living cells start multiplication The ideal gradient factor for starting a culture is not the gradient of optimal cell growth but the lowest gradient factor which will permit cell growth if maximum increment in number is to be obtained before it is necessary to change medium or subculture either of which disturbs the gradient The development of a chemostat like device (10) with a series of automatic controls governing oxygen tension oxidation reduction potential and CO concentration and regulating the rate of medium exchange would seem to be the solution to mass production but it still remains far in the future

Summary

1 Further observations on human cells isolated from the blood of patients with acute monocytic leukemia and now in culture for over 18 months (J 111) and 22 months (J 96) are presented

2 These observations lend further support to the view that the histiocytic and monocytic series are one and the same

3 They emphasize the extreme pleomorphism of cells of this series and illustrate production of multinuclear and mononuclear giant cells of various types Our studies would favor the view that these giant cells result from endomitosis with polyploidy or polyteny rather than from fusion of cells

4 They emphasize the close relationship of monocytic leukemia with reticulum cell sarcoma and Hodgkin s disease

5 A fixed tissue cell does not appear necessary for long term growth of hemic cells

6 Gradient principles appear to be important in all phases of tissue culture and more consideration should be given in our thinking to micro environments and the ecology of cells within the human body as well as in cultures

# 15

# Newer Concepts of Leukopoiesis as Revealed by Leukopheretic and Isotopic Techniques[1]

CHARLES G CRADDOCK JR[2]
University of California at Los Angeles California

The quantitative removal of circulating leukocytes by recirculation of the experimental animal's blood devoid of these cells has helped clarify the interrelationship of reserve and circulating leukocytes. The technique employed has been simple, reproducible, and may be repeated many times without injury to the animal. The details of the procedure (leukopheresis) have been described elsewhere (1).

*If a normal dog's leukocytes are removed rapidly (i e the clearance of* leukocytes from 1½ to 2 blood volumes per hour for 2 to 3 hours) the response shown in Fig 1 is observed. Leukopenia of about 1000 cells/mm[3] is easily attained. It is not possible to lower the count below this point despite prolonged leukopheresis. Initially it was thought this was because the clearance of leukocytes was imperfect and certain numbers were being returned. Subsequent evidence has shown that the inability to reduce this "floor" results from an increased rate of entry of leukocytes during this period. The failure of the leukocyte count to begin to rise immediately after cessation of the procedure is due to an accelerated distribution of leukocytes to the tissues. This period of sustained leukopenia is always observed if marked reduction in circulating

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[1] This work was done in collaboration with Drs John S Lawrence, Seymour Perry, and Irwin Weinstein, with the technical assistance of Miss Mary Baker and Miss Gloria Paul, from the Department of Medicine, UCLA Medical Center and the Hematology Research Laboratory, Wadsworth General V A Hospital, Los Angeles, California. This work was supported in part by grants from Parke Davis & Co and the U S Public Health Service and the Gladys Bowyer Foundation.

[2] Associate Professor of Medicine, on leave of absence with the Armed Forces. Present address U S Army Hospital, Fort Ord, California.

[3] We are indebted to Dr Norman Simmons for the method of DNA extraction.

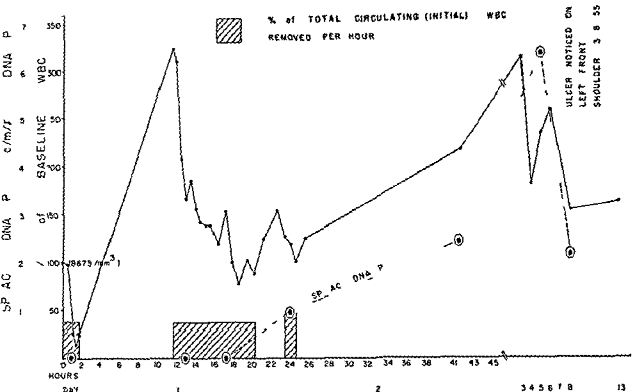



FIG 2 Prolonged leukopheresis in a normal dog instituted during the period of accelerated entry of leukocytes into the circulating blood (phase II) after a previous acute leukopheresis In this case $P^{32}$ was administered at time 0 Note the delayed appearance of cells with labeled DNA Dog 36-62 Continuous exchange—500 μc $P^{32}$ given one hour before exchange Total circulating WBC (initial) 1 909 × $10^{11}$ Total WBC removed 7 694 × $10^{10}$ Wt 46 lbs Blood Vol 2 20 liters Date 2 23 55

cells has been brought about no matter how long the leukopheresis is continued Conversely it is not observed if only moderate degrees of leukopenia are induced

Once the leukocyte level in the circulation begins to rise it does so in a linear fashion reaching a point 150 to 300% above baseline in the next 6 to 8 hours Cells continue to pour into the circulation at this

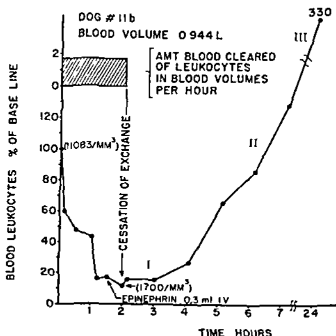



FIG 1 Diagram of typical response to a single acute leukopheresis in normal dogs

greatly augmented rate until a certain maximum (which varies from animal to animal) has been reached If leukopheresis is reinstituted during phase II leukocytes may be removed at greater speeds and in far greater numbers for long periods of time without effecting a serious leukopenia Figure 2 shows such an experiment in which some five times the number of cells circulating are removed during a prolonged leukopheresis (77 billion) The data on specific activity of leukocyte DNA $P^{32}$ shown in this figure further indicate the size of the marrow reserve of mature myeloid leukocytes Thus cells which began to incorporate $P^{32}$ into DNA at time 0 did not begin to appear in the circulation until after the removal of the bulk of cells by leukopheresis This fact will be elaborated on subsequently

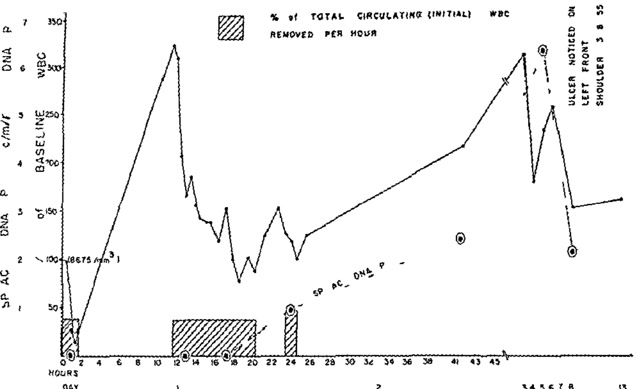



Fig 2 Prolonged leukopheresis in a normal dog instituted during the period of accelerated entry of leukocytes into the circulating blood (phase II) after a previous acute leukopheresis In this case $I^3$ was administered at time 0 Note the delayed appearance of cells with labeled DNA Dog 36-C2 Continuous exchange—500 µc $I^3$ given one hour before exchange Total circulating WBC (initial) 1 909 × $10^{1}$ Total WBC removed 7 694 × $10^{10}$ Wt 46 lbs Blood Vol 2 20 liters Date 2 [illegible]3 55

The leukocytosis occurring after leukopheresis involves neutrophilic leukocytes exclusively Total lymphocyte counts either fall or remain unchanged A finding which was unexpected and hard to reconcile at first was the absence of a left shift in the differential counts even after prolonged and repeated leukopheresis The leukocytosis after leukopheresis involves mature granulocytes Serial bone marrow studies in dogs subjected to repeated leukopheresis revealed a slowly developing

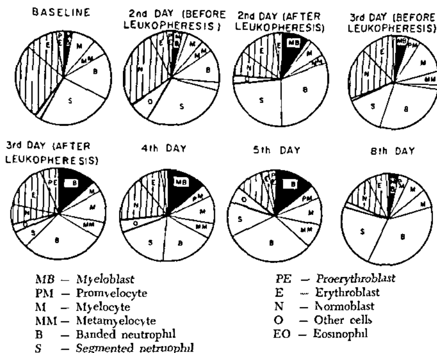



FIG 3 Bone marrow differential cell counts in a normal dog subjected to repeated leukopheresis

myeloid hyperplasia reaching a maximum at 5 days Figure 3 shows this in terms of the M/E ratio These facts together with our inability to deplete normal dogs to the point where they failed to develop a leukocytosis substantiate the concept put forth by Osgood (2) and others (3) that the circulating mass of leukocytes is a small fraction of the total body mass of these cells

It is interesting to compare the response of normal dogs to irradiated animals Figure 4 shows a normal animal leukopheresed on three successive days The repeated development of leukocytosis in fact at an increasingly rapid rate *is apparent Figure 5 shows the response in a*

dog exposed to $LD_{50}$ irradiation 1 hour prior to leukopheresis (250 r) The initial response is similar to the normal In fact close comparison of the initial leukocytosis in many animals reveals it to be identical with the normal when expressed in terms of per cent change from baseline Subsequent responses become less and less adequate Although a rapid rise in leukocyte count may occur the maximum level attained becomes progressively lower This fall in the level of maximum leukocytosis after

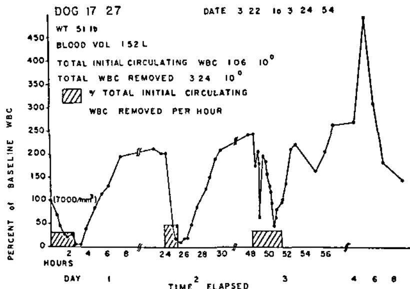



FIG 4 The response in a normal dog to repeated leukopheresis Note that the maximum blood leukocyte level after each leukopheresis is about the same Also note that the final marked leukocytosis occurs gradually over a period of days rather than hours

leukopheresis is of considerable interest and correlates roughly with the marrow content of leukocytes

Figure 6 compares the response in two normal dogs (one receiving large doses of chloromycetin) with a dog exposed to 125 r of whole body x irradiation A characteristic of the normal response to repeated leukopheresis is the slow development of an even greater leukocytosis reaching a maximum on the fifth day (Fig 7) This is the point of maximum marrow myeloid hyperplasia This response which is closely parallel from one normal dog to another is absent in irradiated animals This leukocytosis which occurs after the stimulus to rapid leukocytosis has

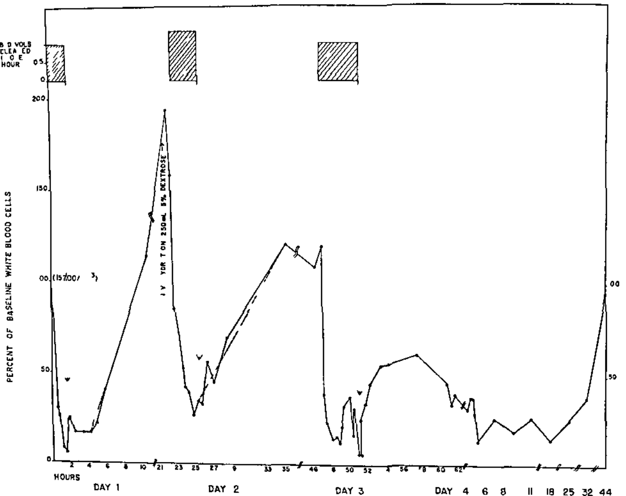



FIG 5 The response in a dog exposed to LD 50 whole body irradiation 1 hour prior to the first leukopheresis (The dose of 250 r was delivered from two tubes equidistant from lateral and central surfaces The dose has been found to represent the LD 50 when given in this way) Dog -3 30 Irradiated—LD 50 Wt 33 lbs Blood Vol 1 247L Date 4 20 to 4 22 54

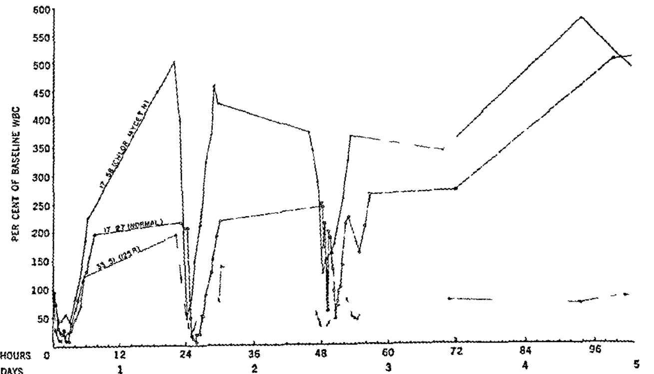



FIG 8 The comparative response in two normal dogs (one receiving chloromycetin which does not affect the normal response) and an animal subjected to 125 r of whole body x irradiation 1 hour before time 0 This dose results in leukopenia and thrombocytopenia about 2 weeks after exposure in the average dog not subjected to leukopheresis Note the absence of delayed leukocytosis in this animal

ceased seems a different phenomenon from the immediate leukocytosis after leukopheresis It involves primarily increased volume of leukocyte production

The leukocyte response to leukopheresis has been spoken of as though it involves cells released from the marrow This conclusion was reached only after the burden of evidence pointed toward this concept One

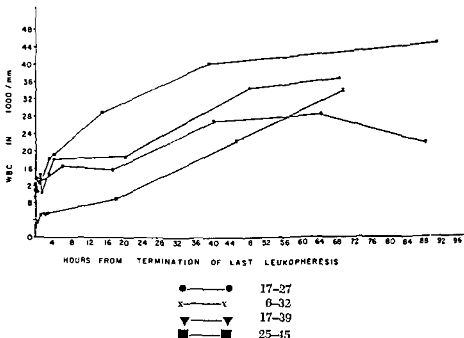



FIG 7 The delayed gradual leukocytosis in four normal dogs after leukopheresis on the three preceding days The starting point here is at the cessation of leukopheresis on the third day

other possible source of leukocytes contributing to the leukocytosis after depletion is redistribution of cells from areas of sequestration as in the spleen and lung Intravenous epinephrine failed to alter the response however Furthermore splenectomized animals behaved as normal as is shown in Fig 8

The other potential source is re entry of leukocytes from extravascular spaces The evidence against this phenomenon is twofold First animals rendered severely leukopenic by x irradiation and repeated leukopheresis (i e Fig 5) when autopsied displayed many leukocytes at areas of inflammatory reaction in the lung and intestine but practically none in the marrow or vascular channels These extravascular leuko

cytes must have migrated to these areas before the animal became leukopenic and failed to re-enter the circulation at least in sufficient numbers to sustain the leukocyte count at even a low level Second isotope experiments point to the marrow as the chief reservoir of mature leukocytes taking part in this phenomenon

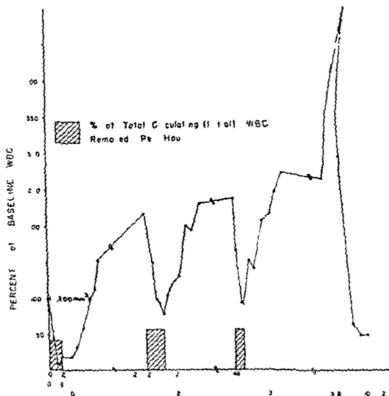



FIG 8 The response of a normal dog splenectomized several months earlier to repeated leukopheresis Dog 11 5 (splenectomized) Total circulating WBC (initial) 1 2 × $10^{10}$ Total WBC removed 3 374 × $10^{10}$ Wt 32 lbs Blood Vol 1 073 liters Date 1 3-55 to 1 5 55

We have employed a method of extraction of leukocyte DNA which in our hands has been far superior to the Schmidt Thannhauser principal[4] The DNA was of a high degree of purity as shown by the chromatograph in Fig 9 The method involves the precipitation of nucleoprotein from isolated leukocyte nuclei by sodium xylene sulfonate

at high concentration and subsequent precipitation of DNA at low concentration and acid pH

In dogs the appearance of leukocytes whose DNA is labeled with $P^{32}$ follows the pattern shown in Fig 10 The maximum specific activity in normal animals is attained between the fourth and fifth day as shown in Fig 11 This means that a period of 3 days elapses after administra-




FIG 9 The comparative purity of leukocyte DNA prepared by the Simmons method (*left*) with a sample of virus DNA prepared by the Schmidt Thannhauser method (*right*)

tion of the isotope before leukocytes mature from the stage where they are capable of incorporating the $P^{32}$ into DNA and move out into the circulation During this time a high differential exists between the labeled marrow leukocytes and the unlabeled peripheral leukocytes Repeated leukopheresis or injections of typhoid bacilli intravenously early in this period will cause a somewhat earlier entry of labeled cells into the circulation (Fig 12) If leukopheresis is performed at the end of this period before the specific activity of DNA phosphorus has risen in the blood leukocytes there is observed a sharp rise in specific activity immediately after the procedure (Fig 13) This indicates that the bulk of cells contributing to the leukocytosis after leukopheresis came from the marrow

The concept of a large marrow reserve of mature leukocytes available for rapid entry into the circulation on demand would seem well established The role of this reservoir in the physiology of leukocytosis is

clearly shown in the response to leukopheresis Acceleration of leuko cyte production by hyperplasia of leukocyte progenitors in the bone marrow required some 3 to 5 days The marrow leukocyte reserve

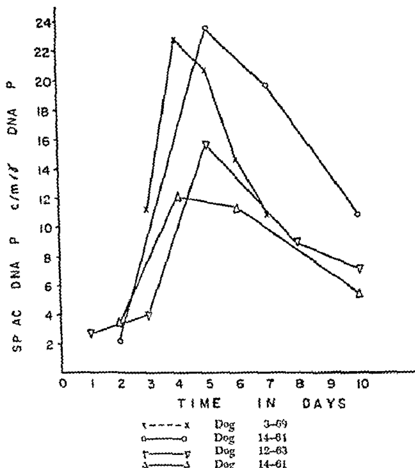



FIG 10 The rate of appearance of leukocytes with P³² labeled DNA in four normal dogs (ordinate expressed as counts per minute per microgram DNA phosphorus)

supplies the need for more cells in the interim before this increased leukopoiesis occurs

Experiments in animals whose marrow leukocyte reserve has been depleted as by previous exposure to x irradiation indicate that the size

at high concentration and subsequent precipitation of DNA at low concentration and acid pH

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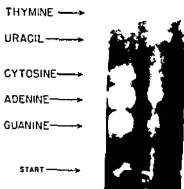



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leukocyte DNA P specific activity indicates that the bulk of cells entering the blood after intravenous typhoid came from the labeled marrow reservoir. It is noteworthy that the rise began during the leukopenic state again indicating that the accelerated release of cells from the marrow had already begun.

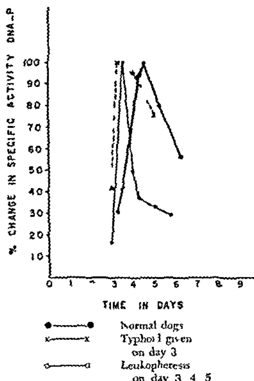



Fig 12 The effect of repeated leukopheresis and typhoid vaccine in accelerating the peak of maximum specific activity of circulating leukocyte DNA P[3]. Here the ordinate is expressed as per cent change in specific activity.

Figure 16 compares the leukocyte response after repeated leukopheresis and that after repeated intravenous typhoid. The general similarity of response is evident as is the sharp increment in specific activity of leukocyte DNA P[3] on the third and fourth days. The lack of significant rise in specific activity on the fifth day is consistent with the fact that the marrow leukocyte reservoir is no longer more highly labeled than peripheral leukocytes.

of the marrow reservoir can be estimated by the response to leuko pheresis (Fig 14) It will be seen that the general response to leukopheresis follows the same pattern in this severely depleted animal as in the normal The height of the maximum leukocyte level attained after the procedure is much lower than in the normal animal however This and other similar experiments suggest that the height of leukocytosis

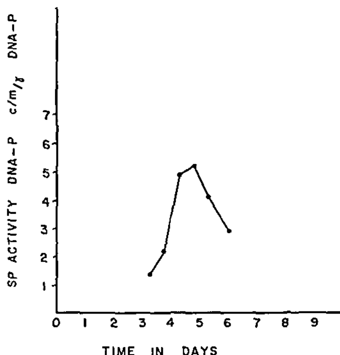



FIG 11 The curve of specific activity of DNA $P^{32}$ in a normal dog with observations made at close intervals during the period of maximal specific activity The peak specific activity is on the afternoon of the fourth day

after leukopheresis depends on the numerical size of the marrow reservoir

Recent data obtained in our laboratory by my associates Drs Seymour Perry and Irwin Weinstein indicate that other methods of producing transient leukopenia followed by leukocytosis cause the release of cells from the marrow reservoir of leukocytes Figure 15 shows the response of the blood leukocytes to intravenous typhoid vaccine The leukopenia followed by a rapidly developing leukocytosis is in most experiments remarkably similar to the pattern after leukopheresis The rapid rise in

leukocyte DNA P³² specific activity indicates that the bulk of cells entering the blood after intravenous typhoid came from the labeled marrow reservoir It is noteworthy that the rise began during the leukopenic state again indicating that the accelerated release of cells from the marrow had already begun

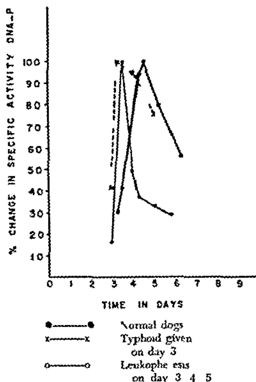



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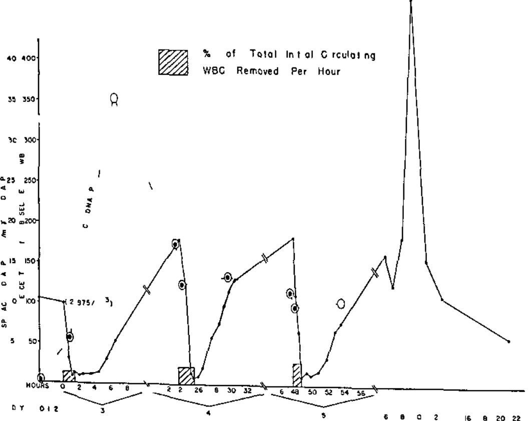



Fig 13 Leukopheresis initiated at the end of the period when a high differential exists between marrow and blood leukocyte DNA $P^{3}$ specific activity The sharp rise in blood leukocyte DNA $P^{3}$ after the initial leukopheresis indicates the circulating cells have come from the marrow Dog 20 72 Given 500 μc $P^{3}$ three days before exchange Total circulating WBC (initial) 2 917 × $10^{10}$ Total WBC removed 2 450 × $10^{10}$ Wt 34 lbs Blood Vol 1 67 liters Date 5 16 55 to 5 19 55

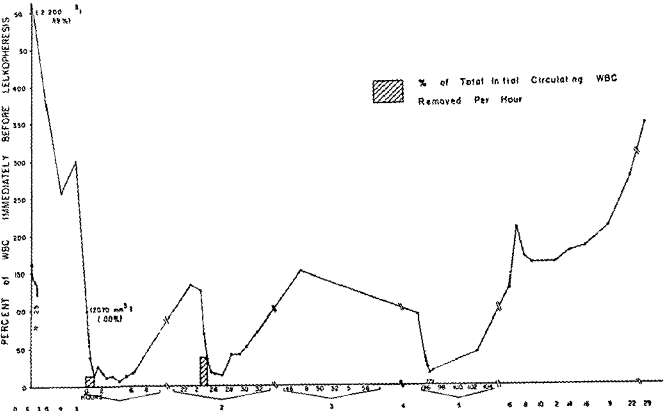



FIG 14 The response to repeated leukopheresis in a dog rendered leukopenic by previous exposure to 125 r of whole body irradiation Note that the 100% leukocyte count = 2070 cells/mm³ Dog 35 71 Irradiated with 125 R 15 days before exchange Total circulating WBC (initial) 0 33 × 10⁹ Total WBC removed 0 165 × 10⁹ Wt 35 lbs Blood Vol 1 6 liters Date 5 5 55 to 5 9 55

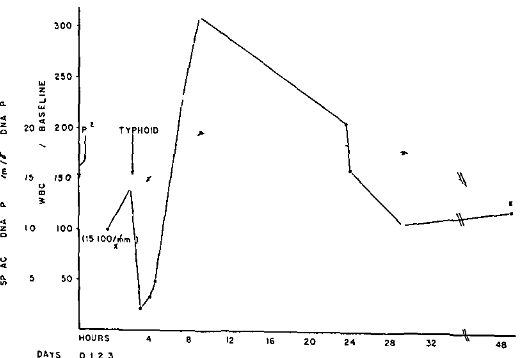



Fig 15 The response to intravenous typhoid of a normal dog given 500 μc of P32 3 days earlier Note the similarity of both leukocyte count and DNA P32 specific activity to the response after leukopheresis

From these data it appears probable that the size of the human marrow leukocyte reserve may be susceptible to study by modification of this type of approach Such knowledge may have value in the management of patients receiving agents which suppress leukopoiesis and in the study of altered leukopoiesis in disease

It is of interest to compare the large reserve of mature leukocytes in the marrow with the small reserve of platelets Removal of all circulating platelets by a similar procedure produces a thrombocytopenia which persists for 4 days before a gradual rise begins This sustained *thrombocytopenia is associated with disappearance of platelets in the* marrow and hyperplasia of immature megakaryocytes These cells require a period of 3 to 4 days for maturation to the point of platelet production This limited reserve of platelets has considerable clinical significance since massive replacement with platelet poor blood will result in prolonged thrombocytopenia

The mechanisms involved in this release of white cells from the marrow at varying rates depending on peripheral demand are unknown Humoral influences have not yet been detected in our experiments The *primary stimulus in leukopheresis seems to be the removal of cells from* the circulating blood The entire pattern of response resembles a redistribution of cells from the central marrow toward the periphery Some sort of tissue continuity must exist between the leukocytes throughout the body so that removal of cells in the periphery promotes a shift of cells from the marrow reservoir

## REFERENCES

1 Craddock C G Jr Adams W S Perry S Skoog W A and Lawrence J S *J Lab Clin Med* 45 881 (1955)
2 Osgood E E *Blood* 9 1141 (1954)
3 Yoffey J M *Ann N Y Acad Sci* 59 928 (1955)

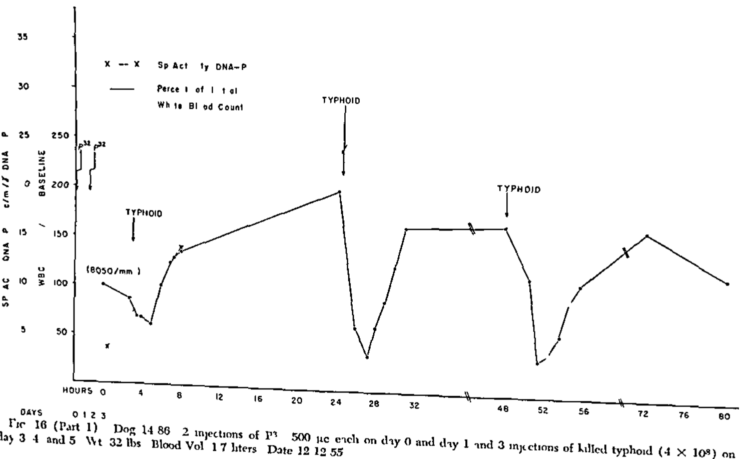



Fig 16 (Part 1) Dog 14 86 2 injections of P³² 500 μc each on day 0 and day 1 and 3 injections of killed typhoid (4 × 10⁸) on day 3 4 and 5 Wt 32 lbs Blood Vol 1 7 liters Date 12 12 55

Pt 16 (Part 2) Dx 38 70 500 µc 1^s given at 0 time Total circulating WBC (initial) 1 8 × 10^10
Total WBC removed 3 06 × 10^10 Blood Vol 2 2 liters Date 4 18 55

16

# Endocrine Influences on Blood Formation[1]

ALBERT S GORDON GEORGE J FRUHMAN
and CHARLES D SIEGEL

Department of Biology Graduate School of Arts and Science
New York University New York New York

It is now conceded that the endocrine glands constitute a link in the chain of physiologic factors that exert a regulative influence on the processes of blood formation and destruction This relation might have been anticipated since the hormones as is well known significantly affect the rate and sometimes even the quality of a wide variety of biological processes and it would be expected that these actions should be imparted either directly or indirectly to the blood forming organs It would seem inadvisable in this report to discuss in detail again the evidence supporting a relation between the endocrine system and the blood elements for this topic has been reviewed on several occasions (13 15 16 32 33 34) Most of the recent attention has focused on the effects of manipulation of the pituitary adrenal axis on the peripheral leukocyte numbers Thus it is now clear that administration of adrenal cortical steroids particularly those oxygenated at C 11 or activation of the adrenal cortex by ACTH or as a result of stress almost invariably evokes a peripheral leukocytic response characterized by relative and absolute lymphopenia eosinopenia and neutrophilia (11 17 40) The processes responsible for this triad of response have not been resolved fully and it is apparent that the mechanisms differ to some extent depending on whether the treatment is applied acutely or over a sustained period of time In the relatively short term experiments the suppressive effects of the adrenal principles on the numbers of peripheral eosino

[1] Work done during the tenure of a grant in aid (project No GS 434) from the National Science Foundation

Fellow of the Damon Runyon Memorial Fund Present address Department of Anatomy Albert Einstein College of Medicine Yeshiva University Bronx New York

phils and lymphocytes *may involve at least two pathways (1) a re* distribution of these elements to or a sequestration within vascular and extravascular tissue sites (56 57 74) and (2) a destructive action on these cells accomplished in the tissues and perhaps also in the body fluids (18 51 52 60) When administered over protracted periods of time impairment is noted in the production of both eosinophilic and lymphocytic elements (1 32 33) On the other hand the peripheral polymorphonuclear leukocytosis an almost invariable component of the response to corticoid or ACTH treatment is probably the result at least in part of an *increased production of these elements within the bone* marrow (55 73)

It was the lymphocytic suppressing properties of the adrenal factors that probably first suggested their use in the treatment of animal blood dyscrasias Thus Murphy and Sturm (48) observed the ability of adrenal cortical factors and pituitary extracts containing adrenocorticotropic activity to inhibit the growth of transplanted lymphatic leukemic cells in rats Similarly adrenalectomy was found to increase greatly the sus ceptibility of rats to this type of transplantable tumor (66) Along the same lines favorable results were reported with cortisone in inhibiting the growth of malignant lymphoid tumors in mice (39)

Reports soon appeared attesting to the ability of ACTH and cortical steroids to induce remissions in leukemic states in human beings (24 53) It is now recognized that ACTH and the oxysteroids are useful mem bers *in the armamentarium available for the palliative treatment of* such diseases as chronic lymphatic leukemias lymphosarcomas and acute myeloid leukemias The mechanism of action of the pituitary and adrenal factors in these situations is not as yet known fully In the lymphatic leukemias it seems probable that the ameliorative effects must stem to some extent from the destructive (catabolic) effects of the steroids on the lymphoid growths as well as from the inhibition of the anabolic re actions responsible for the synthesis of both cytoplasmic and nuclear components important in the fabrication of lymphoid cell structure Cor rection of a maturation defect in the granulocytic elements probably mediated indirectly may provide the basis for whatever beneficial effects accrue with the cortical factors in the acute myelogenous leukemias It has been suggested that the steroids may operate by mobilizing factors such as metabolites or enzymatic components which are lacking in the leukemic state (29 54)

A discussion of the mechanisms of hormone action in cellular prolifera tive processes should include the ability of endocrine factors to alter certain fundamental aspects of metabolism Thus adrenalectomy in the

rat results in a significant increase in the glycolytic activity of the bone marrow associated with an increase in the myeloid erythroid ratios and in the relative immaturity of the granulocytic forms (32 33) Treatment with adrenal cortical extract lowers the rate of glycolysis and restores the morphological features of the marrow to normal Since chemical changes must of necessity precede the morphological ones in cellular differentiation processes it is possible that the ameliorative effects of the steroids in leukemia may derive from their capacity to modify abnormal metabolic activity (e g glycolytic respiratory ratios) in the blood forming cells The adrenal may accomplish this by regulating the blood flow and therefore the delivery of oxygen and nutrients to the cells Attention should be given to the possibility that adrenalectomy by establishing tissue ischemia sparks a glycolytic mechanism within the blood forming organs that is conducive to excessive leukocytic and diminished erythrocytic proliferation

Basic to the understanding of the influence of hormones in leukemia would be a knowledge of the reasons why greater success is obtained in children afflicted with the acute myeloid leukemias and why refractoriness to these agents develops in most cases despite their continued administration A study of the intermediary metabolism undergone by the steroids in the refractory patients might shed light on the nature of the resistance The liver and the adrenal undoubtedly participate in this phenomenon for it is well recognized that these organs operate under both normal and pathological conditions in converting active steroids into others with little or no physiological activity Worth the effort and time of the chemist would be the development of specific active congeners of the adrenal steroids that might help to defeat the adaptive enzymatic mechanisms which emerge during the continued use of such agents (12)

In a recent symposium on leukemia research there was unanimity of opinion among the groups of international participants that a fuller comprehension is required of the basic mechanisms underlying the processes of blood cell growth differentiation and release from the blood forming organs before deeper and more significant inroads can be made into the solution of the problem of leukemia It is our opinion in this regard that greater energies should be expended in exploring further the effects of naturally occurring substances on hemopoietic processes for it is possible that a change in the balance of these factors might provide the soil for the conversion of a normal cellular type into one with leukemic potentialities

The use of endocrine stimuli in hematologic studies has been made

by us not only as a means of investigating hormonal influences on blood formation a problem *important in its own right* but also *as an effective* tool in uncovering basic mechanisms that might be concerned in the production distribution and ultimate disposal of the blood cells It has become evident to us as well as to others that in studies of blood formation a knowledge of the numbers of circulating blood elements although informative does not necessarily afford an accurate indication of the events transpiring within the blood forming organs Thus the agent being tested could conceivably induce changes in plasma volume or cause a shifting of cells into and out of the circulation phenomena which would render false impressions of the actual hemopoietic events Moreover studies by Yoffey (72) and by Osgood (49) demonstrate that the circulating white cells represent only a small fraction of the total leukocytic population of the body

The present research is concerned primarily with a quantitative approach to the effects of hormones given alone and in combination with a potent antimetabolite on the cellular elements of bone marrow A modification of a method devised for assessing the numbers of marrow cells in the rat (26 33) has been employed At the termination of the experiments the animals were subjected to light ether anesthesia exsanguinated by cardiac puncture and decapitated The femurs or tibias *were dissected quickly trimmed completely of all surrounding* tissue and split lengthwise A sample representing the greater part of the available marrow was drawn into a pipet calibrated to contain exactly 10 or 20 mm $^3$ The marrow was expelled into a test tube containing 2 ml of homologous serum and a suspension was formed by drawing the marrow up and down in a rubber nippled glass pipet Samples were then taken for hemacytometer counts and for smears One sample was *diluted with Randolph s phloxine methylene blue mixture to determine* the total numbers of nucleated cells and of eosinophils By means of myelogram percentages obtained from stained smears the numbers of the other cell types were computed Nucleated erythroid cells were identified from marrow smears treated by Ralph s benzidine technique and counterstained with Harris hematoxylin Wright stained smears were also examined In the present studies the results of the marrow analyses are thus expressed in *terms of concentration* (*i e cells per cubic millimeter* of marrow)

## Effects of 17 Hydroxycorticosterone (Cortisol) in Adrenalectomized Rats

In previous papers (26 33) we have described the effects of adrenalectomy and steroidal factors on hemopoiesis Thus at 5 days after

adrenalectomy in the rat there occurred approximately a 50% increase in the total numbers of eosinophils and lymphocytes and a 50% reduction in the numbers of nucleated red cells in the femoral marrow. Daily injections of 2 to 6 mg of cortisol for 7 days induced a marked lowering in the numbers of eosinophils and lymphocytes within the marrow. Neutrophilic cellular numbers were depressed with the 4 and 6 mg doses of this steroid. Accompanying these effects on leukocytic numbers were oppositely induced changes in the nucleated erythroid cell population. Daily amounts of cortisol as small as 0.5 mg, which are anabolic in the adrenalectomized rat, resulted in more than a doubling, and quantities of 2 to 6 mg (catabolic doses) evoked approximately a 300% increase in the total numbers of nucleated erythrocytes. The marrow changes induced by cortisol were accompanied by peripheral eosinopenia, lymphopenia, and neutrophilia. Reticulocytosis and rises in both the hematocrit and hemoglobin values were also in evidence.

### Effects of Cortisol and Growth Hormone in Hypophysectomized Rats

Somewhat similar actions were induced by cortisol in hypophysectomized rats. Here 1 mg amounts of the steroid administered daily for 2 weeks resulted in peripheral eosinopenia, lymphopenia, and neutrophilia (Fig. 1). A diminution in total white cell count was also noted

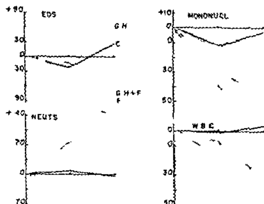



Fig. 1. Per cent changes at 1 and 2 weeks in peripheral eosinophils (EOS), lymphocytes and monocytes (MONONUCL), neutrophils (NEUTS), and total white cell counts (WBC) in hypophysectomized rats receiving growth hormone (GH, 7 animals) ———, cortisol (F, 9 animals) - - - - - - -, growth hormone and cortisol (GH + F, 7 animals) – – – – –, and controls (C, 10 animals) ——— which received the vehicle for the hormones. Data from Fruhman and Gordon (27).

which was attributable to the decrease in the numbers of lymphocytes the predominant circulating leukocyte in the rat Growth hormone (Raben Westermeyer preparation) given in daily doses of 0 5 mg for the same 2 week period caused a tendency toward peripheral eosinophilia and neutropenia a marked reticulocytosis was also present Growth hormone administered together with cortisol tended to inhibit to some extent the effects exerted by cortisol alone on the peripheral leukocytic picture Repeated injections of cortisol induced a 70% reduction in eosinophils and a 50% decrease in the numbers of lymphocytes within the bone marrows of hypophysectomized rats (Figs 2 and 3) These changes probably were responsible for the peripheral

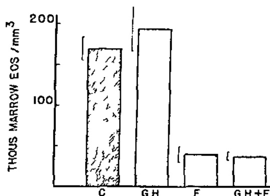



FIG 2 Mean eosinophil values (thousands/mm 3) within the femoral marrows of the four groups of rats shown in Fig 1 Two dots ( ) indicate values significantly different from controls ($P < 0.01$) Vertical lines to the left of the bars represent ± 1 standard error of the mean Data taken from Fruhman and Gordon (27)

eosinopenia and lymphopenia noted in these animals Extramedullary sources may account for the maintained peripheral neutrophilia in the face of a reduction in the numbers of the elements in the marrows of rats treated chronically with cortisol Growth hormone when administered along with cortisol was without influence on the course of the marrow lymphopenia and eosinopenia caused by the steroid alone The growth hormone was strongly erythropoietic in the hypophysectomized rat causing approximately a 70% increase in the concentrations of nucleated red cells within the bone marrow (Fig 4) In the dosages employed cortisol exerted no significant effects on the concentrations of nucleated red cells but since the total cellularity suf

fered a reduction in the animals treated with this steroid the percentages of the nucleated red cells were necessarily increased (Fig 5)

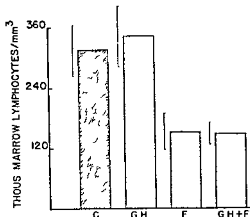



Fig 3 Mean lymphocyte values (thousands/mm³) within the femoral marrows of the four groups shown in Fig 1 Symbols are the same as for Fig 2

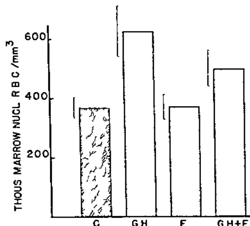



Fig 4 Mean nucleated RBC values (thousands/mm³) within the femoral marrows of the four groups of rats shown in Fig 1 Symbols are the same as for Fig 2

#### Effects of a 2 4 Diaminopyrimidine in Intact Rats

More recently our attention has been directed to a quantitative study of the effects of antimetabolites on the blood forming processes in the rat Our initial study centered on the peripheral and marrow effects of

a diaminopyrimidine namely 2 4 diamino 5 (3 4 dichlorophenyl) 6 ethylpyrimidine (50 276 Wellcome Research Laboratories) This is a member of a group of pyrimidines shown originally by Hitchings and his associates (41) to be *competitive antagonists of folic acid for the growth of Lactobacillus casei* The hematological effects of some of these compounds have been examined recently in the dog and rat (38)

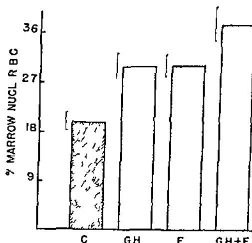



FIG 5 Mean percentages of nucleated RBC within the femoral marrows of the four groups of rats shown in Fig 1 Symbols are the same as for Fig 2

In our experiments intraperitoneal injections of 2 mg of 50 276 daily for 5 days into adult male rats weighing approximately 350 g resulted in a decrease in the numbers of circulating reticulocytes lymphocytes eosinophils and neutrophils This antimetabolite also decreased significantly the total nucleated cell population of the marrow a reflection largely of the reductions in numbers of neutrophilic and lymphoid elements within this organ Normal and sham operated rats injected daily *with 50 276 (6 mg/kg initial body weight) for more than 30 days* gained weight and appeared healthy Adrenalectomized animals treated in the same manner died within a week

## Effects of 50 276 and Cobalt in Intact Rats

The use of antimetabolites in the treatment of leukemic states is often handicapped by their tendency to depress erythropoiesis *It seemed* conceivable to us that if a potent erythropoietic agent were to be given *simultaneously* with the antimetabolite the erythropoietic depressing effects of the latter could be circumvented while still achieving the de

sired suppressive action on the leukocytes To test this possibility in organic cobalt was employed This metal has been shown by many workers to exert marked erythropoietic effects in intact as well as in endocrine deficient animals (4 10 35 43 67)

Four groups of adult male rats (8 in each group) in the 350-g body weight range were established The first received 2 mg of 50-276 intra peritoneally the second was injected subcutaneously with 2 mg of cobalt chloride the third with the combination of 50-276 and cobalt and the fourth with 1% saline the vehicle for these agents The substances were administered on five occasions over a 6-day period and all animals were killed on the seventh day The cobalt treated rats displayed a moderate s imulation of erythropoiesis as seen from the reticulocytosis and in creased hematocrit percentages hemoglobin values and red cell counts The numbers of nucleated red cells within the marrows were also sig nificantly elevated by this substance On the other hand, no significant changes were noted in the numbers of peripheral or bone marrow leukocytes

To our surprise instead of cobalt s overcoming the effects of the anti metabolite on the erythroid elements a synergistic depressive action was manifested not only on these but also on other of the cellular types of the marrow Figure 6 depicts the marked decrease in the numbers of nucleated red cells within the marrow evoked by the combination an effect not in evidence with the antimetabolite alone The action on splenic erythropoiesis was also striking Here the antimetabolite given singly was without effect on the nucleated red cell percentages within the spleen whereas cobalt induced a great increase in their numbers On the other hand the combination produced a marked lowering in the percentages of these elements In addition neutrophilic and eosinophilic elements virtually disappeared from both the circulation and bone mar row (Figs 7 and 8)

Table III indicates that cobalt induced a slight drop in body weight In this series body weights were not influenced significantly by 50 276 given alone The combination of cobalt and the antimetabolite resulted in a marked decrease in body weight considerably greater than seen with 50 276 administered singly These results have been reported in preliminary form (28)

This study prompted us to investigate the question as to whether the actions of the antimetabolite could be modified by hormonal factors known to exert hemopoietic effects in endocrine deficient animals These included 11 dehydro 17 hydroxycorticosterone (cortisone) growth hor mone testosterone and a plasma filtrate obtained from anemic rabbits

a diaminopyrimidine namely 2 4 diamino 5 (3 4 dichlorophenyl) 6 ethylpyrimidine (50 276 Wellcome Research Laboratories) This is a member of a group of pyrimidines shown originally by Hitchings and his associates (41) to be competitive antagonists of folic acid for the growth of *Lactobacillus casei* The hematological effects of some of these compounds have been examined recently in the dog and rat (38)

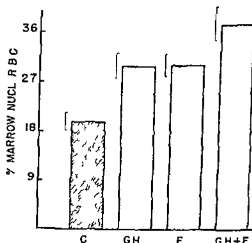



FIG 5 Mean percentages of nucleated RBC within the femoral marrows of the four groups of rats shown in Fig 1 Symbols are the same as for Fig 2

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## Effects of 50 276 and Cobalt in Intact Rats

The use of antimetabolites in the treatment of leukemic states is often handicapped by their tendency to depress erythropoiesis It seemed conceivable to us that if a potent erythropoietic agent were to be given simultaneously with the antimetabolite the erythropoietic depressing effects of the latter could be circumvented while still achieving the de

of both neutrophilic and mononuclear elements Administration of 5 mg doses of cortisone also resulted in a moderate decrease in leukocyte count due almost completely to the characteristic lowering of lymphocyte numbers eosinophil levels were also depressed Neutrophils displayed the usual elevation in numbers after cortisone treatment the combination of 50 276 and cortisone resulted in depressive effects on the total leuko

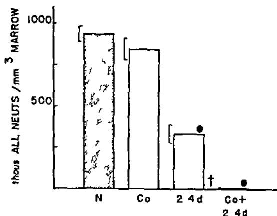



Fig 7 Mean total neutrophilic cell values (thousands/mm 3) within the tibial marrows of the four groups of rats shown in Fig 6 Symbols are the same as in Fig 6

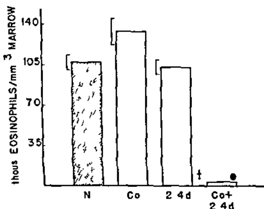



Fig 8 Mean eosinophil values (thousands/mm 3) within the tibial marrows of the four groups of rats shown in Fig 6 Symbols are the same as in Fig 6

In these experiments rats weighing approximately 200 g were employed and a dose of 50 276 was sought which would evoke only moderate changes in bone marrow cellularity This was found to be 1 2 mg per 200 g rat per day After treatment signs of the pernicious anemia type of bone marrow with "megaloblasts" and giant myelocytes were in evidence The numbers of mitotic figures also were increased significantly in both the erythroid and myeloid series probably a manifestation of

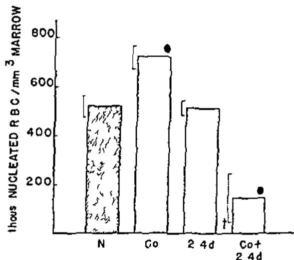



FIG 6 Mean nucleated RBC values (thousands/mm³) within the tibial marrows of four groups of rats injected five times over a 6 day period with (1) cobalt (Co) (2) 50–276 (2 4d) (3) cobalt + 50–276 (Co + 2-4d) and (4) the vehicle for these agents (N) One ellipse (●) indicates values significantly different from controls ($P < 0\ 01$) One cross (†) indicates that the value for the combination is significantly different from that for the antimetabolite alone ($P < 0\ 01$) Vertical lines to the left of the bars represent ± 1 standard error of the mean

arrested development The design of the experiment was similar to that described above for the cobalt antimetabolite series Subcutaneous injections of the hormonal factor were given separately and in combination with the antimetabolite which was administered intraperitoneally to groups of rats Controls were injected with the vehicles for these agents Injections were given five times over a 6 day period and all rats were killed on the seventh day

## Effects of 50 276 and Cortisone in Intact Rats

### PERIPHERAL LEUKOCYTE COUNTS

It will be noted from Table I that 50 276 induced approximately a 25% decrease in the leukocyte count due to a diminution in the numbers

of both neutrophilic and mononuclear elements Administration of 5 mg doses of cortisone also resulted in a moderate decrease in leukocyte count due almost completely to the characteristic lowering of lymphocyte numbers eosinophil levels were also depressed Neutrophils displayed the usual elevation in numbers after cortisone treatment the combination of 50 276 and cortisone resulted in depressive effects on the total leuko

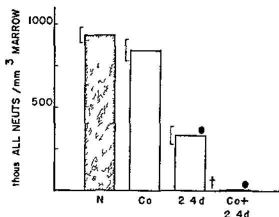



FIG 7 Mean total neutrophilic cell values (thousands/mm 3) within the tibial marrows of the four groups of rats shown in Fig 6 Symbols are the same as in Fig 6

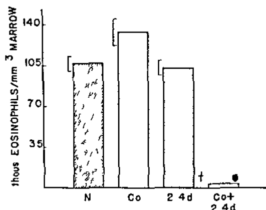



FIG 8 Mean eosinophil values (thousand /mm 3) within the tibial marrows of the four groups of rats shown in Fig 6 Symbol are the same as in Fig 6

TABLE I

EFFECTS OF 50–276 CORTISONE (E) AND GROWTH HORMONE (GH) ON THE PERIPHERAL WHITE CELL VALUES IN INTACT RATS

| Treatment† | Total WBC thousands/mm³ | Mononuclears thousands/mm³ | Neutrophils thousands/mm³ | Eosinophils numbers/mm³ |
|---|---|---|---|---|
| Controls (6) | 20 5 ± 1 0 | 16 7 ± 1 0 | 3 2 ± 0 5 | 319 ± 50 |
| 50–276 (6) | 15 4 ± 1 9‡ | 13 5 ± 0 5‡ | 1 9 ± 1 7 | 324 ± 132 |
| 5 mg E (6) | 16 6 ± 1 9 | 7 1 ± 0 8§ | 9 5 ± 1 0§ | 40 ± 36§ |
| 50–276 + 5 mg E (6) | 7 0 ± 1 5§ ‖ | 1 9 ± 1 1§ ‖ | 5 1 ± 0 9‖ | 22 ± 1§ |
| Controls (6) | 15 9 ± 2 9 | 12 5 ± 2 6 | 3 2 ± 0 8 | 210 ± 71 |
| 50–276 (6) | 9 2 ± 0 8 | 8 5 ± 0 7 | 0 5 ± 0 1§ | 90 ± 52 |
| GH (6) | 15 4 ± 2 8 | 11 9 ± 1 6 | 2 6 ± 0 7 | 329 ± 82 |
| 50–276 + GH (6) | 11 2 ± 2 0 | 9 7 ± 2 2 | 0 9 ± 0 4‡ | 106 ± 82 |

The experiments with cortisone and with growth hormone were run at different times and therefore are indicated separately with their own controls

† Numbers of animals are indicated in parentheses under Treatment All figures represent means ± standard errors

‡ Indicates $P < 0\ 05$ for the values compared to the controls

§ Indicates $P < 0\ 01$ for the values compared to the controls

‖ Indicates $P < 0\ 01$ for the values of the combinations compared to 50–276 alone

cyte values lymphocytes and eosinophils greater in magnitude than those seen with either material administered alone Neutrophil numbers however were intermediate between those noted with the two agents given separately

BONE MARROW

In determining the myelograms for the animals receiving the antimetabolite the various cellular series examined included abnormal as well as normal elements Thus the total neutrophil" category comprised all neutrophilic elements including giant myelocytes and metamyelocytes The "mature neutrophil" group consisted only of cells that were polymorphonuclear and in which neutrophilic granulation was evident and the cytoplasmic basophilia was no longer present The erythroid category included both megaloblastic" and normoblastic elements Unclassified cells composed of "blasts" plasmacytes macrophages mast cells megakaryocytes unidentified elements and destroyed forms were not listed separately but were included in the total nucleated cell counts

Table II indicates the effects of the various agents studied on the concentrations of the cellular elements of the marrow It will be observed that 50 276 produced a decrease in the cellularity of the marrow mainly due to a drop in neutrophilic and lymphocytic elements Nucleated red cell and eosinophilic numbers were not affected significantly by this dose of antimetabolite Cortisone exerted little effect in the intact rat save to produce a rise in the concentrations of mature neutrophilic forms and a lowering in the eosinophil numbers in the 5 mg injected group The marrows of the rats receiving the combination of the antimetabolite and the 5 mg dose of cortisone revealed a greater lowering in the total cellularity and in eosinophil numbers than that noted with 50 276 given alone A synergistic depressive action was observed with the combination on the neutrophilic cell category an effect especially apparent on the mature neutrophilic forms

In the rats displaying the potentiated depressive effects the marrows were very reddened grossly and fluid in consistency The hypocellular structure was infiltrated by numerous mature erythrocytes Many late normoblasts were seen Increased numbers of mitotic figures possibly in arrest and fragmented nuclei also were apparent Giant myelocytes were less conspicuous The general impression gained was that the major morphological defect resided in the nucleus rather than in the cytoplasm As judged by staining both with Wright s stain and with the Ralph benzidine hematoxylin technique the cytoplasm was often more

TABLE II

EFFECTS OF 50–276 AND VARIOUS AGENTS ON THE CELLULAR CONCENTRATIONS WITHIN THE MARROWS OF INTACT RATS

| Treatment† | Total nucleated cells millions/mm³ | Nucleated red blood cells millions/mm³ | Total neutrophils millions/mm³ | Mature neutrophils millions/mm³ | Lymphocytes millions/mm³ | Eosinophils thousands/mm³ |
|---|---|---|---|---|---|---|
| Controls (18) | 2 72 ± 0 07 | 0 56 ± 0 06 | 0 80 ± 0 08 | 0 25 ± 0 03 | 0 39 ± 0 05 | 121 ± 11 |
| 50–276 (9) | 2 04 ± 0 10‡ | 0 53 ± 0 12 | 0 58 ± 0 06§ | 0 16 ± 0 02§ | 0 18 ± 0 03‡ | 100 ± 9 |
| 2 5 mg E (6) | 2 88 ± 0 18 | 0 55 ± 0 10 | 0 95 ± 0 13 | 0 38 ± 0 06 | 0 44 ± 0 08 | 131 ± 33 |
| 50–276 + 2 5 mg E (6) | 2 02 ± 0 13‡ | 0 32 ± 0 11 | 0 64 ± 0 06 | 0 23 ± 0 04 | 0 16 ± 0 02‡ | 73 ± 12‡ |
| 5 mg E (6) | 2 70 ± 0 07 | 0 68 ± 0 12 | 0 93 ± 0 06 | 0 50 ± 0 07‡ | 0 28 ± 0 07 | 74 ± 19§ |
| 50–276 + 5 mg E (6) | 1 28 ± 0 27‡ ‖ | 0 35 ± 0 08§ | 0 24 ± 0 04‡ ‖ | 0 06 ± 0 002‡ ‖ | 0 31 ± 0 07 | 27 ± 7‡ ‖ |
| 2 mg testosterone (6) | 2 27 ± 0 08‡ | 0 52 ± 0 06 | 0 70 ± 0 09 | 0 22 ± 0 06 | 0 32 ± 0 07 | 48 ± 5‡ |
| 50–276 + 2 mg testosterone (6) | 2 15 ± 0 17§ | 0 56 ± 0 17 | 0 49 ± 0 10§ | 0 13 ± 0 06 | 0 29 ± 0 05 | 48 ± 3‡ ‖ |
| HP (6) | 3 10 ± 0 08‡ | 0 66 ± 0 03 | 0 93 ± 0 14 | 0 27 ± 0 07 | 0 41 ± 0 06 | 127 ± 17 |
| 50–276 + HP (6) | 2 44 ± 0 17 | 0 75 ± 0 08 | 0 60 ± 0 04§ | 0 07 ± 0 009‡ ‖ | 0 25 ± 0 08 | 105 ± 16 |
| 2 mg phenylhydrazine (6) | 2 98 ± 0 17 | 1 37 ± 0 19‡ | 0 59 ± 0 03§ | 0 20 ± 0 06 | 0 22 ± 0 05§ | 98 ± 17 |
| 50–276 + 2 mg phenylhydrazine (6) | 1 92 ± 0 15‡ | 0 96 ± 0 13‡ ¶ | 0 27 ± 0 08‡ ‖ | 0 05 ± 0 02‡ ‖ | 0 11 ± 0 04‡ | 64 ± 7‡ ‖ |
| 50–276 (9) | 1 99 ± 0 14‡ | 0 56 ± 0 11 | 0 56 ± 0 07§ | 0 11 ± 0 03‡ | 0 19 ± 0 04‡ | 92 ± 9 |
| 1 mg GH (9) | 2 73 ± 0 08 | 0 68 ± 0 11 | 0 87 ± 0 04 | 0 31 ± 0 06 | 0 32 ± 0 05 | 149 ± 36 |
| 50–276 + 1 mg GH (9) | 1 53 ± 0 14‡ ¶ | 0 40 ± 0 07 | 0 30 ± 0 06‡ ¶ | 0 04 ± 0 01‡ ¶ | 0 13 ± 0 04‡ | 73 ± 14§ |

The data for GH are indicated separately because these experiments were run at a different time Another group of 50–276 injected rats run simultaneously with the GH series is included

† Numbers of animals are indicated in parentheses under Treatment All figures represent means ± standard errors E = cortisone GH = growth hormone HP = plasma filtrate

‡ Indicates P < 0 01 for the values compared to the controls

§ Indicates P < 0 05 for the values compared to the control

‖ Indicates P < 0 01 for the values of the combination compared to 50–276 alone

¶ Indicates P < 0 05 for the val[illegible] of the combination compared to 50–276 [illegible]

"mature (hemoglobiniferous) than might have been expected from the nuclear appearance

BODY AND SPLEEN WEIGHTS

*As seen from Table III both 50 276 and the 2 5 mg* doses of cortisone permitted gains in body weight to occur but at a somewhat inhibited rate The 5 mg levels of cortisone resulted in a slight loss in body weight A loss in body weight was also sustained by the rats receiving the combination of 50 276 and the 2 5 mg doses of cortisone An even greater reduction of weight was evident in the rats injected with the combination of 50 276 and the higher doses of cortisone

A tendency toward a decrease in splenic weights was seen with the antimetabolite and with the 5 mg levels of cortisone The combination of the antimetabolite and the 5-mg levels of cortisone produced greater decreases in splenic weights than those noted with either agent administered singly

A moderate hemorrhage amounting to 1 5% of body weight was applied to test the erythropoietic potential of rats that had been injected with the antimetabolite and the 5-mg levels of cortisone for 7 days Hematocrit percentages and body weights were followed at 1 4 and 7 days after the bleeding All 24 rats survived the entire experimental period All groups experienced a small drop in hematocrit at the first day after the bleeding This attained its lowest level at 4 days in the controls and in the groups injected with 50 276 The rats injected with cortisone or the combination tended to recover more quickly At 7 days the hematocrit values of the four groups were not significantly different At 4 days the control animals and those which had received the 5-mg doses of cortisone were manifesting weight gains Little change in weight was noted in the rats receiving the combination whereas those treated with 50-276 alone experienced a sharp reduction in weight By the seventh day however gains in weight were noted in all four groups

Effects of 50 276 and Growth Hormone in Intact Rats

PERIPHERAL LEUKOCYTE COUNTS

The only significant effect produced by daily 1 mg doses of beef growth hormone (GH) (Armour No M108) *was a rise in the circulating* eosinophil numbers (Table I) The total leukocyte count and mononuclear and neutrophil numbers were not affected No evidence was observed for a synergistic action of GH and the antimetabolite on the numbers of any of the leukocytic types examined

TABLE III

EFFECTS OF 50–276 AND VARIOUS AGENTS ON BODY WEIGHT IN THE INTACT RAT

| Treatment† | Mean weights before treatment g | Mean weights after treatment g | Differences ± standard errors |
|---|---|---|---|
| Controls (8) | 359 | 359 | 0 ± 4 |
| 50–276 (8) | 340 | 330 | − 10 ± 6 |
| 2 mg cobalt (8) | 337 | 325 | − 12 ± 4‡ |
| 50–276 + 2 mg cobalt (8) | 332 | 276 | − 56 ± 6§ ‖ |
| Controls (21) | 202 | 230 | + 28 ± 3 |
| 50–276 (12) | 205 | 217 | + 12 ± 3§ |
| 2 5 mg E (6) | 220 | 231 | + 11 ± 4§ |
| 50–276 + 2 5 mg E (6) | 209 | 201 | − 8 ± 2§ ‖ |
| 5 0 mg E (6) | 202 | 196 | − 6 ± 4§ |
| 50–276 + 5 0 mg E (6) | 202 | 183 | − 19 ± 3§ ¶ |
| 2 mg testosterone (6) | 208 | 231 | + 23 ± 2 |
| 50–276 + 2 mg testosterone (6) | 208 | 222 | + 14 ± 2§ |
| 1 mg GH (9) | 193 | 229 | + 36 ± 2‡ |
| 50–276 + 1 mg GH (9) | 200 | 210 | + 10 ± 4§ |
| HP (6) | 197 | 238 | + 41 ± 4‡ |
| 50–276 + HP (6) | 199 | 227 | + 28 ± 5¶ |
| 2 mg phenyl hydrazine (6) | 201 | 215 | + 14 ± 2§ |
| 50–276 + 2 mg phenyl hydrazine (6) | 191 | 181 | − 10 ± 3§ ‖ |

The experiments with cobalt were conducted on heavier animals and therefore are indicated separately with their own controls

† Numbers of animals are indicated in parentheses under Treatment E = cortisone GH = growth hormone HP = plasma filtrate

‡ Indicates $P < 0.05$ for the values compared to the controls

§ Indicates $P < 0.01$ for the values compared to the controls

¶ Indicates $P < 0.05$ for the values of the combinations compared to 50–276 alone

‖ Indicates $P < 0.01$ for the values of the combinations compared to 50–276 alone

### Bone Marrow

Table II reveals that GH did not influence significantly the numbers of any of the cellular types of the marrow in the intact rat Nevertheless this factor potentiated the depressive action of 50 276 on the numbers of total nucleated cells Again it was the drop in the numbers of neutrophilic cells that contributed mostly to this effect

### Body Splenic, and Thymic Weights

Daily administration of 1 mg of GH evoked body weight gains of 30 ± 4 g over a period of 7 days in test hypophysectomized rats averaging 178 g in weight. In the intact rats utilized in this study significant increases in body weights over those of the vehicle treated controls were observed with GH (Table III) This hormone also induced increases in thymic ($P<0.05$) but not in splenic weights No augmented depressive effects were noted with the combination of GH and 50 276 on the body splenic or thymic weights

### Effects of 50 276 and Testosterone in Intact Rats

Eosinopenia was the only action evident in the marrows of rats that received daily injections of 2 mg of testosterone propionate (Table II) When given with 50 276 it was conspicuously without effect in altering the marrow changes induced by the antimetabolite alone In addition it failed to modify the body (Table III) and splenic weights when administered alone or together with the antimetabolite

### Effects of 50-276 and a Plasma Filtrate Obtained from Anemic Rabbits in Intact Rats

Previous reports (3 6 36 37) have indicated considerable erythropoietic activity in boiled filtrates of acidified plasma secured from rabbits rendered severely anemic by phenylhydrazine Thus highly significant increases in peripheral red cell numbers and in hemoglobin hematocrit and reticulocyte values accompanied by both morphologic and enzymatic evidence of increased erythropoietic activity of the bone marrow are observed after repeated injections of this material for approximately 2 weeks into intact or hypophysectomized rats

We considered it of interest to determine the effects of this filtrate (HP) and the antimetabolite on the blood picture Daily injections of 1 ml of HP corresponding to 2.66 ml of original plasma were made into intact rats either alone or in combination with the 1 2 mg doses of 50 276 Materials were given as in previous groups for 5 days Table II indicates that HP induced a significant increase in the total nucleated cellularity of the marrow This rise was probably the result of the com

bined small increases in the numbers of cellular types especially the nucleated red cell and neutrophilic forms Considerably greater erythropoietic activity is evoked by HP when administered over a longer period than 5 days (6 36 37)

The total marrow cellularity in the rats administered the combination was intermediate between that observed for either agent given alone A potentiated depressive effect with the combination of 50 276 and HP on the marrow cells was noted in the mature neutrophilic series This was not apparent for the nucleated red cells lymphocytes or eosinophils

Body weight gains were increased significantly by the administration of HP (Table III) When given with 50 276 HP tended to restore the body weight gains to the levels characteristic of the vehicle injected controls No significant influence of HP injected alone or with the antimetabolite was noted on the splenic weights

**Effects of 50 276 and Phenylhydrazine in Intact Rats**

In this series of experiments intact rats were administered 2 mg of phenylhydrazine alone and in combination with 50 276 daily for 5 days in the same manner as conducted in all the previous experiments Phenylhydrazine given singly resulted in approximately a 15% drop in peripheral hematocrit values but the antimetabolite by itself exerted no effects on this parameter The combination however evoked approximately a 50% fall in hematocrit Reticulocyte values were elevated to 46% by phenylhydrazine an intermediate value of 29% was evident with the combination

Within the marrow phenylhydrazine induced highly significant increases in the concentrations of nucleated red cells but caused a fall in the numbers of total neutrophils (Table II) No significant effects were exerted on the eosinophil numbers Joint administration of the antimetabolite with phenylhydrazine resulted in a greater depression in the numbers of neutrophils and eosinophils than that seen with either agent given alone The combination of the two factors did not result in a synergistic depression of the nucleated erythroid cells In fact the concentrations of these elements were significantly higher than those noted in either the controls or in the rats receiving 50 276

The rats *treated with phenylhydrazine failed to gain weight* at the same rate as in the controls (Table III) The combination of phenylhydrazine and 50 276 caused a loss of body weight not apparent with either agent alone

**Discussion**

The results of the present as well as previous studies indicate clearly that endocrine deficiency states and hormonal treatments exert a significant influence on blood forming processes It would seem that the endocrine deficient rat is a more sensitive test object than the intact rat for assessing the hemopoietic actions of the hormones Thus the quantities of steroid hormone and the length of time the treatment must be administered to induce eosinopenia lymphopenia and erythropoietic actions in the marrow are significantly greater in the intact animal than in either adrenalectomized (26) or hypophysectomized (27) rats Thus comparable treatment with adrenal cortical steroids evokes considerably greater increases in the numbers of nucleated red cells in the marrows of adrenalectomized as compared to intact rats A similar situation prevails for the effects of growth hormone and testosterone as judged by their ability to speed erythropoiesis in the hypophysectomized animal (32 31) This greater response of the deficient animal to endocrine replacement may reside in an increased sensitivity of its tissues to the prevailing levels of the hormone or to a decreased rate of destruction of the hormone caused by the generally lowered metabolic state of the animal

Of considerable interest was the capacity of certain hormonal and other agents to potentiate the actions of a 2 4 diaminopyrimidine (50-276) an antagonist of folic acid metabolism on some of the cellular values of the blood and bone marrow When judged on the basis of numbers the cellular series within the marrow most affected by the antimetabolite was the neutrophilic with increasing resistance being noted for the eosinophilic and lymphocytic elements The synergistic depressive effects with the combinations were also revealed in approximately the same order indicating that the provocative agent may operate by increasing the effectiveness of the antimetabolite on cellular proliferation and maturation It would appear that the erythroid cells were more resistant to the actions of the antimetabolite than the granulocytes In this regard it has been demonstrated that leukocytic divisions are associated with reduplication of chromosomes and continued deoxyribose nucleic acid (DNA) synthesis As Dustin (19) has remarked this is not so evident for the divisions of the erythrocyte especially in the later stages where there appears to be a diminution in the quantity of DNA in the erythroblastic nuclei Such mitoses without DNA synthesis would be expected to be more resistant to antagonists of folic acid metabolism

Of the agents used cobalt was the most effective in augmenting the

actions of the antimetabolite The combination led to an almost complete disappearance of the neutrophilic elements especially mature forms as well as eosinophils from the bone marrow Erythroid cell concentrations which were increased by cobalt alone and not affected by the antimetabolite in the dosage employed were greatly depressed by the combination A similar enhanced depressive action on the numbers of marrow neutrophils eosinophils and nucleated erythroid forms was produced by the combination of 50 276 and cortisone No synergistic action with cortisone was noted on the neutrophils of the peripheral circulation however indicating that the neutrophilia attending cortisone treatment is probably not totally a result of a stimulatory action on myeloid cell proliferation within the marrow but is also due to an ability of adrenal steroids to mobilize this cellular type from extramedullary sites At the same time such results and others along the same lines in the present study point up an already emphasized fact (32 33 49) that the peripheral cell numbers do not necessarily reflect quantitatively or even qualitatively the alterations occurring within the blood forming organs The data indicate that pretreatment with cortisone and the antimetabolite did not influence the recovery of rats from a single bleeding Thus at 7 days after the hemorrhage normal hematocrit values were reestablished and gains in body weight were occurring Future experiments will be conducted to determine the effects of maintained treatment with these agents on the response to repeated bleedings Another facet of this work will examine the leukocytic regenerative capacities in such animals

The results with growth hormone were particularly interesting in that they revealed this agent as able to potentiate the effects of the antimetabolite in lowering neutrophilic cell numbers within the marrow whereas GH by itself did not produce significant effects on this cellular line Similarly the depressive action exerted on the eosinophil numbers by the combination of GH and the antimetabolite was not evident when each of these factors was administered independently

A somewhat different pattern was unfolded with the hemolytic substance phenylhydrazine and with the plasma filtrate obtained from rabbits made anemic by this agent Each material given in combination with the antimetabolite produced greater depressive effects on the mature marrow neutrophils than was seen with either agent alone No such action was apparent however on the nucleated red cell population with the combination of phenylhydrazine and 50 276 or HP and this antimetabolite In fact the concentrations of nucleated red cells obtained with the combination were greater than those seen with the anti

metabolite alone a point indicating different levels of action of the various provocative agents used on the cellular types of the marrow A protective action of HP on x irradiated rats has been reported recently (65)

The lymphocytic population of the marrow consistently did not appear to participate in the synergistic inhibitory actions This suggests that all marrow lymphocytes may not be formed in this organ or that they represent extramedullary elements that have infiltrated the marrow as a result of the structural damage inflicted by the antimetabolite

Instances of enhanced depressive actions exerted by combinations of antimetabolites and hormones on cellular proliferative processes and general viability have appeared in the literature Thus Farber and his associates (23) have concluded that more satisfactory results in the treatment of leukemia in children were obtained when cortisone or ACTH was used in combination with antimetabolites Shapiro (61) reported the ability of stilbestrol but not testosterone to increase the inhibitory action of 8 azaguanine on transplantable mammary carcinoma in mice On the other hand testosterone was found to increase the toxicity of aminopterin in mice whereas estrogen was ineffective in this regard (68) Along the same lines Goldin *et al* (31) found aminopterin to be more lethal in male than in female mice The present experiments however indicate that when testosterone was combined with 50-276 enhanced inhibition was not apparent on the body weights or on any of the parameters studied within the bone marrow Our results with testosterone are not necessarily at variance with those of others It seems likely that the dosage employed and the time over which the agent is administered are vital factors in influencing the response of the animal In addition it is possible to envision at least three different patterns of response depending on whether treatment with the agent is given days before simultaneously with or days after exposure to the antimetabolite

The precise nature of the mechanism by which the various agents employed in this study produce their potentiating effects remains speculative It is commonly accepted that the inhibitory actions of antimetabolites are based on their ability to compete with the metabolite for specific intracellular receptors This is largely a dosage phenomenon with the more antimetabolite supplied the larger the quantity of metabolite displaced (5) and the greater the amount of the latter required to overcome the inhibitory effects In the instance of folic acid those cells with the greatest requirement for this metabolite would be most influenced by antagonists of folic acid metabolism This appears to be the case for the cellular elements of the marrow which are avid in their demand for

this metabolite Thus one mode of action is that the potentiating agent may operate by increasing the incorporation of the antimetabolite into the marrow an effect which may be due to a change in cellular permeability If this were so it might be anticipated that the administration of a greater quantity of the antimetabolite would reproduce the effects of the synergistic combination This appears possible for some of the agents employed such as cortisone and cobalt but would not operate for others such as the growth factor This hormone evoked enlargement of the thymus caused most likely by lymphocytic hyperplasia (25) If the above theory were applicable universally it would be expected that the combination of GH and the antimetabolite should have resulted in synergistic depressive effects on the thymus which was not the case

An alternative concept involving enzymatic pathways finds support in the observation by Dinning *et al* (14) that the reduction in marrow cellularity induced by aminopterin in chickens is associated with a loss in choline oxidase from this organ The suggestion was made by them that the antifolic may operate by displacing folic acid from a position in the choline oxidase system Metallic cobalt also has been found to inhibit this enzyme complex (44) Since as the present study indicates cobalt markedly potentiates the action of 50 276 on the various blood parameters examined a possible mechanism of the synergism may be in the inhibition of choline oxidase or other enzyme systems

Another but related mechanism would implicate the provocative agent as augmenting the need for specific nutrients which are required by the cell for proliferation Thus Stoerk (64) has found that treatment with testosterone or cortisone discouraged more strongly the growth of a transplanted lymphosarcoma in pyridoxine deficient rats than in animals on a complete diet Similar augmented inhibitory actions were found with cortisone on the growth of a lymphosarcoma in riboflavin deficient mice (20) Moreover the combination of deoxypyridoxine or flavotin (a riboflavin analog) and 8 azaguanine inhibited the growth of a mammary carcinoma in mice to a greater extent than did the antimetabolite alone (62) Of interest also is the finding that cortisone injections into rats resulted in approximately a 100% increase in the excretion of vitamin $B_{12}$ tagged with $Co^{60}$ after administration of a test dose of this vitamin (7) Similarly it is reported (46) that vitamin $B_{12}$ can overcome the catabolic effects of cortisone Large doses of cortisone also lead to loss of vitamin A from the livers and kidneys of rats (8) Growth hormone another of the agents employed in the present studies is known to be ineffective in increasing body weights in animals deficient in vitamin $B_{12}$ (7) vitamin A (21) or pyridoxine (2) and induces a rapid deple

tion of pantothenic acid in rats maintained on a pantothenic acid deficient diet (45) Thus the hormones may act by increasing the need for vitamins which serve as cofactors for enzymes It is evident therefore that instead of invoking one locus of action for both the antimetabolite and the potentiating agent the possibility may be justified that they operate at different levels which are reinforcing in their inhibitory effects In this connection *in vitro* techniques such as those employed by Weisberger *et al* (69) might serve to differentiate between direct and indirect actions of the agents employed

Other possibilities would mark the potentiating factor as an inhibitor of a system which is concerned with the inactivation or excretion of the antimetabolite or as an evocator of a metabolic change which converts the antimetabolite into a more potent inhibitor Isotopically labeled antimetabolites would help in the resolution of these questions

It has been observed in the present experiments that body weight losses often accompanied the depressive effects on the marrow If this action is to be interpreted as a "toxic" one then it becomes important to determine its basis Pertinent here would be a knowledge of the sites of production of the "toxins" and their loci of action On the other hand situations are encountered in which the provocative agent may enhance the depressive influence of the antimetabolite on the bone marrow in animals continuing to display good increases in body weight (e g HP)

The primary thought underlying the present experiments was to uncover combinations of antimetabolites and hemopoietic stimulants that might selectively depress specific cell lines of bone marrow with a minimum of undesirable side effects This has not been accomplished totally as perhaps might have been anticipated from the widespread activities of both the antimetabolite and the various stimulants used The best hope for achieving a potentiated inhibitory effect on a cell type would appear to depend on the ability of the agent to affect along with the antimetabolite an essential system specific for that particular cell line There may be agents which can accomplish this Well exemplified is the case of the tropic principles of the adenohypophysis which act by and large only on their specific target organs In this connection growth hormone has been reported to stimulate not to influence or even to inhibit growth in different organs within the same animal (47) Of even greater interest are the observations that inhibition and stimulation can occur within different parts of the *same* organ with a given hormone or hormone combination Thus Weiss and Rossetti (71) have reported stimulation of growth of ganglion cells in the brain of amphibian larvae bearing transplants of thyroid tissue or thyroxin soaked agar placed

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above the 4th ventricle One pair of cells however concerned with larval swimming showed shrinkage in size Weiss (70) has attributed this to a different biochemical constitution of these cells which predisposed them to react to thyroid hormone with a growth response of opposite sign from that of the other cells of the group Similarly growth hormone in combination with estrogen inhibited cartilage development but acted in a synergistic manner in increasing bone proliferation within the tibia (59) These observations justify a more intensive search for humoral factors that will operate on specific cell lines Evidence is available for the existence of circulating specific erythropoietic factors (6 22 30 36 37 42) and possible leukopoietic principles (9 63) The testing of these compounds when obtained in pure form along with the antimetabolites would be most important It would also be profitable to attempt the synthesis of the analogs of these "hemopoietins" and to examine them for their depressive effects on blood formation

The concept has also been advanced (50 58 70) that in addition to stimulating factors other basic humoral substances exist which restrain growth within the animal These appear to be organ specific (58) and there may be factors operative for each of the blood cellular lines (50) It is not inconceivable that such inhibitors are chemical analogs of the stimulatory agents in which case the extent of cellular proliferation would be a function of the relative amounts of the two sets of principles Such inhibitors if isolated would constitute a natural source of hemopoietic depressants

It is not outside the realm of credulity to consider that endocrine factors as they circulate within the organism play a role in growth processes by controlling the metabolism and availability to the specific cell lines of the naturally occurring inhibitors and accelerators It is our feeling that reasoning and research patterned along these lines will lead to a deeper understanding of the mechanisms underlying growth and maturation in normal and pathological cells

## Summary

Some aspects of the relation of the endocrine system to blood formation are presented By employing a quantitative method for assessing the numbers of the different cellular types within the bone marrow of the rat it was found that adrenalectomy resulted in increases in the numbers of lymphocytes and eosinophils accompanied by reductions in the numbers of nucleated erythroid cells within this organ Treatment with 17 hydroxycorticosterone (cortisol) induced a diminution in the numbers of lymphocytes and eosinophils in the marrows of both adrenal

ectomized and hypophysectomized rats The nucleated red cell population of the marrow in adrenalectomized rats was increased by this steroid Growth hormone given together with cortisol inhibited partially the peripheral lymphopenia eosinopenia and neutrophilia induced by cortisol alone *In the dosage used however it was without effect in* overcoming the marrow changes caused by cortisol

A 2 4 diaminopyrimidine (50 276 Wellcome) administered in moderate dosage to intact rats produced a significant decrease in the numbers of circulating reticulocytes neutrophils eosinophils and lymphocytes The marrow displayed megaloblast like cells and giant myelocytes as well as an increased number of mitotic figures probably representing cells in division arrest Neutrophilic and lymphocytic cellular numbers in the marrow were lowered by this substance The influence of various agents both hormonal and nonhormonal in nature was examined on the action of this antimetabolite Inorganic cobalt demonstrated to be a potent erythropoietic agent produced when administered with 50 276 a marked depression in the numbers of nucleated red cells and neutrophilic and eosinophilic elements of the marrow Similar effects on the neutrophilic and eosinophilic components of marrow were noted when cortisone was given along with the antimetabolite Growth hormone potentiated the depressive action of the antimetabolite on the marrow neutrophilic elements Testosterone was without effect A plasma filtrate obtained from rabbits rendered anemic by phenylhydrazine significantly increased bone marrow cellularity in intact rats The combination of the filtrate and 50 276 resulted in a greater lowering in the numbers of mature marrow neutrophils than was evident with the antimetabolite alone Phenyl hydrazine induced a striking increase in the numbers of nucleated red cells and a diminution in the numbers of total neutrophils and lymphocytes within the marrow When it was administered with 50 276 a marked lowering occurred in the numbers of marrow neutrophils lymphocytes and eosinophils Marrow erythropoietic activity however remained significantly above control levels

Several possible mechanisms are proposed for the potentiated depressive effects produced by the various agents employed These envision the provocative agent as acting by (*1*) augmenting the incorporation of the antimetabolite into the affected cells (2) inhibiting a system possibly enzymatic in nature concerned in cellular proliferation (*3*) increasing the need for or metabolism of an essential nutrient or (*4*) enhancing the inactivation or excretion of the antimetabolite The concept is *advanced that hormones may act within the body by regulating the* availability to the different cell lines of specific naturally occurring agents which inhibit or accelerate growth and maturation processes

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REFERENCES

1 Baker B L Ingle D J and Li C H *Am J Anat* **88** 313 (1951)
2 Beare J Beaton J R Smith F and McHenry E W *Endocrinology* **52** 396 (1953)
3 Benard H Dantchev D and Gajdos A *Compt rend soc biol* **148** 1950 (1954)
4 Berlin N I *Acta Haematol* **5** 1 (1951)
5 Bethell F H Swenseid M E Myers M C and Sturgis C C *Proc 3rd Intern Congr Hematol* 331 (1951) Abstract
6 Borsook H Graybiel A Keighley G and Windsor E *Blood* **9** 734 (1954)
7 Chow B F *in* "Symposium on Protein Metabolism p 14 Nutrition Symposium Series No 8 The National Vitamin Foundation New York 1954
8 Clark I and Colburn R W *Endocrinology* **56** 232 (1955)
9 Craddock C G Adams W S Perry S Skoog W A and Lawrence J S *J Lab Clin Med* **45** 881 (1955)
10 Crafts R C *Blood* **7** 863 (1952)
11 Dalton A J and Selye H *Folia Haematol* **62** 397 (1939)
12 Danielli J F *in* "Leukemia Research" p 263 Ciba Foundation Symposium Little Brown & Co Boston 1954
13 Daughaday W H Williams R H and Daland G A *Blood* **3** 1342 (1948)
14 Dinning J S Keith C K Davis P L and Day P L *Arch Biochem* **27** 89 (1950)
15 Dougherty T F *Physiol Revs* **32** 379 (1952)
16 Dougherty T F and Dougherty J H *Ann Rev Physiol* **15** 195 (1953)
17 Dougherty T F and White A *Endocrinology* **35** 1 (1944)
18 Dougherty T F and White A *Am J Anat* **77** 81 (1945)
19 Dustin P Jr *in* Leukemia Research" p 244 Ciba Foundation Symposium Little Brown & Co Boston 1954
20 Emerson G A Wurtz E and Zanetti M E *Federation Proc* **9** 357 (1950)
21 Ershoff B H and Deuel H J Jr *Endocrinology* **36** 280 (1945)
22 Erslev A *Blood* **8** 349 (1953)
23 Farber S Downing V Schwachman H Toch R Appleton R Heald F King J P and Feriozi D *Proc 2nd Clin ACTH Conf* 251 (1951)
24 Farber S Schwachman H Toch R Downing V Kennedy B H and Hyde J *Proc 1st Clin ACTH Conf* 328 (1950)
25 Feldman J D *Anat Record* **110** 17 (1951)
26 Fruhman G J and Gordon A S *Endocrinology* **57** 711 (1955)
27 Fruhman G J and Gordon A S *Acta Haematol* **15** 249 (1956)
28 Fruhman G J Morse B S and Gordon A S *Am J Physiol* **183** 618 (1955) Abstract
29 Furth J *Blood* **6** 964 (1951)

30 Cley P D lor J and Laur C M Compt rend soc biol 148 780 (1954)
31 Goll n A Goldberg B Ortega L G and Schoenbach E B Cancer 2 857 (1949)
32 Gordon A S Recent Progr Horm ne Research 10 339 (1954)
33 Gordon A S Ann N Y Acad S i 59 907 (1955)
34 Gordon A S and Charipper H A Ann N Y Acad Sci 48 615 (1947)
35 Gordon A S Kadow P C Finkelstein G and Charipper H A Am J Med Sci 212 385 (1946)
36 Gordon A S Piliero S J Kleinberg W and Freedman H H Proc Soc Exptl Biol Med 86 255 (1954)
37 Gordon A S Piliero S J Tannenbaum M and Siegel C D Proc Soc Exptl Biol Med 89 246 (1955)
38 Hamilton L Philips F S Sternberg S S Clarke D A and Hitchings G H Blood 9 1062 (1954)
39 Heilman F R and Kendall E C Endocrinology 34 416 (1944)
40 Hills A G Forsham P H and Finch C A Blood 3 755 (1948)
41 Hitchings G H Elion G B VanderWerff H and Falco E A J Biol Chem 174 765 (1948)
42 Hodgson G and Toha J Blood 9 299 (1954)
43 Kleinberg W Am J Physiol 108 545 (1934)
44 Le y H Levison V and Schade A L Arch Biochem 27 34 (1950)
45 Lotspeich W D Proc Soc Exptl Biol Med 73 85 (1950)
46 Meites J Proc Soc Exptl Biol Med 78 692 (1951)
47 Mirand E A Anat Record 120 776 (1954) Abstract
48 Murphy J B and Sturm E Science 99 303 (1944)
49 Osgood E E Blood 9 1141 (1954)
50 Osgood E E Ann N Y Acad Sci 59 806 (1955)
51 Padawer J and Gordon A S Endocrinology 51 52 (1952)
52 Padawer J and Gordon A S Proc Soc Exptl Biol Med 80 581 (1952)
53 Pearson O H Eliel L P Rawson R W Dobriner K and Rhoads C P Cancer 2 943 (1949)
54 Pearson O H Li M C Maclean J P Lipsett M B and West C D Ann N Y Acad Sci 61 393 (1955)
55 Quittner H Ward N Sussman L N and Antopol W Blood 6 513 (1951)
56 Romani J D Compt rend soc biol 146 1665 (1952)
57 Romani J D Compt rend soc biol 146 1680 (1952)
58 Rose S M Ann N Y Acad Sci 60 11 6 (1955)
59 Selye H in "The Hypophyseal Growth Hormone Nature and Actions" p 5 McGraw Hill Book Company New York, 1954
60 Selye H in Stress Acta Inc Montreal 1950
61 Shapiro D M Cancer Research 12 713 (1952)
62 Shapiro D M and Gellhorn A Cancer Research 11 35 (1951)
63 Steinberg B and Martin R A Am J Physiol 161 14 (1950)
64 Stoerk H C Proc Soc Exptl Biol Med 74 799 (1950)
65 Stohlman F Jr and Brecher G Proc Soc Exptl Biol Med 91 1 (1956)
66 Sturm E and Murphy J B Cancer Research 4 384 (1944)
67 Waltner K and Waltner K Klin Wochschr 8 313 (1929)
68 Weintraub S Kraus S D and Wright L T Proc Soc Exptl Biol Med 74 609 (1950)

69 Weisberger A S Suhrland L G and Seifter J *Blood* **11** 1 (1956)
70 Weiss P in "The Hypophyseal Growth Hormone Nature and Actions" p 3 McGraw Hill Book Company New York 1954
71 Weiss P and Rossetti F *Proc Natl Acad Sci U S* **37** 540 (1951)
72 Yoffey J M *Ann N Y Acad Sci* **69** 928 (1955)
73 Yoffey J M Ancill R J Holt J A G Owensmith B and Herdan G *J Anat* **88** 115 (1954)
74 Yoffey J M Metcalf W K Herdan G and Nairn V *Brit Med J* **1** 660 (1951)

# Added Comment[1]

THOMAS F DOUGHERTY

College of Medicine Department of Anatomy
University of Utah Salt Lake City Utah

I should like to open this brief discussion by emphasizing a point of view concerning the influence of certain adrenal cortical steroids on lymphopoiesis In order to save time I shall summarize that which is considered to be fairly definitely proved by the work in our laboratory as well as conclusions drawn from the work of others

First of all the dramatic effect of the C 11 oxygenated corticosteroids *on the destruction of lymphocytes has been considered to be of singular* importance It should be pointed out that in the very first description *of this event attention was drawn to the fact that only the smaller mature* lymphocytes of lymphatic organs and those found in the blood were *lysed by increased amounts of adrenal cortical steroids (3) Immature* cells of the lymph nodes particularly do not show marked morphological *evidence of destruction (3) This does not mean that biochemical al* terations of some sort may not have been induced in these cells Never*theless even overwhelming amounts (fatal in 16 days of treatment) of* adrenal steroids do not produce cellular destruction of reticular lympho*cytes (2) A second and most important point which was also em* phasized (3) was that there is a definite suppression of mitosis of *lymphocytes This early observation has been strengthened by the* fact that it has been shown that there is a linear dose response relation*ship between the amount of hormone and the depression of the number* of mitotic figures in the thymus (4) This was suggested as an assay *method but the amount of work involved limits its usefulness*

An additional and probably important finding with respect to hormonal regulation of lymphopoiesis is that heteroplastic differentiation is inhibited as long as large doses of either adrenal cortical steroids or

[1] Original work reported in collaboration with Martha L Berliner and David L Berliner

ACTH are administered to the experimental animal (2) Heteroplasia is rapidly resumed on cessation of hormone treatment (2)

In addition a considerable amount is known about the structure activity relations among the various adrenal cortical hormones with respect to the processes summarized above (5) First unsaturation of ring A is essential Second an α hydroxyl group at position 17 is essential and unlike the situation for the anti inflammatory effect a β hydroxyl group at position 11 markedly enhances lymphoid suppressing activity Further as we now know reduction of the C 20 position also eliminates lymphocyte suppressing activity (unpublished observations) That alterations in the substitutions of the phenanthrene nucleus produce markedly different degrees of potency is illustrated by the fact that linear regressions of lymphatic organs are obtained by graded doses of these hormones so that comparisons of potency ratios can be made (5) Further evidence from this laboratory is accumulating which indicates that structural differences in these hormones may be related to different effects on lymphatic cells

With respect to lymphatic leukemia we have long stressed the point that adrenal cortical hormones which influence lymphopoiesis also may produce similar effects on lymphatic leukemic cells It has also been apparent however that a greater amount of hormone is required per unit leukemic cell to produce effects similar to those found in normal lymphatic tissue Among the normal lymphatic cells only reticular lymphocytes appear to have similar degrees of resistance The operational point of view with respect to the study of lymphatic leukemia thus emphasizes the differences in response of normal lymphatic tissue cells and lymphatic leukemia cells to adrenal cortical hormones having similar structural characteristics I wish to emphasize that the same hormones which influence lymphopoiesis also affect other cells Also therefore all the changes produced in treatment of lymphatic leukemia may not be ascribed to the effects outlined here The most direct approach to this problem however is to study the relationship of adrenal hormones to the rates of differentiation multiplication and death of lymphocytes which are processes influenced by these hormones in the normal course of events (1) In essence then an important problem with respect to lymphatic leukemia resolves itself into the question of why normal and lymphatic leukemia cells differ with respect to their responses to adrenal cortical steroids

The investigations which are briefly to be described here are those of Drs Martha and David Berliner Time does not permit a complete account of these studies nor can I go into the details of the methods used

Publications in press will deal with these results

The following experiments were performed with lymphatic leukemic and normal lymphocytes Radioactive cortisol labeled in the C 4 position was incubated with malignant cells and normal lymphocytes It was found that the malignant cell catabolizes cortisol and converts it to at least five different steroid hormones *in vitro* The normal lymphocyte has no such capacity and at no time have any metabolites been observed from normal lymphocytes (with the exception of small amounts of cortisone)

*Cortisol 4 $C^{14}$ at zero time* when incubated with normal spleen demonstrates only the cortisol peak on the paper chomatogram (Fig 1) Therefore the extraction procedure did not affect the molecule of cortisol Similarly if the normal spleen is incubated for 4 to 6 hours no other peaks of radioactivity are found When the spleens of AK mice having lymphatic leukemia were incubated for 6 hours however we found five different steroid hormone products Through a brilliant series of investigations by the Berliners these have now been identified They found for example that the products of metabolism are Reichstein s substance U cortisone Reichstein s substance E and 11 $\beta$ hydroxy androstenedione (See Fig 2)

Normal lymph nodes (2 g incubated with 50 $\gamma$ 4 $C^{14}$ cortisol) for 6 hours did give a very small amount of cortisone but none of the other metabolic products Suspensions of cells from AK mice having leukemia produced the same metabolites as cells from their spleens

The question as to whether this is a general metabolic property of lymphatic leukemia cells was investigated by incubating 4 $C^{14}$ labeled cortisol with suspension of cells taken from spleens or lymph nodes of AK mice bearing transplanted lymphatic leukemia cells The same metabolic products were isolated in three different experiments using cells from three different donors with spontaneously occurring lymphatic leukemia Also cells taken from areas of subcutaneous tumor growths of transplanted cells behaved in the same fashion It appears then that mouse acute lymphatic leukemic cells of AK strain have the property of catabolizing cortisol in the same fashion Lymphatic leukemic cells taken from buffy coats of three different patients also showed the same metabolic products Equivalent numbers of buffy coat cells from patients *having infectious lymphocytosis* did not catabolize cortisol *in vitro* Although this metabolic property of lymphatic leukemic cells seems to be constant it appears from preliminary evidence that the human myelogenous leukemia cells and cells of the Gardner lymphosarcoma do not display the same metabolic pathways as those demonstrated for lym

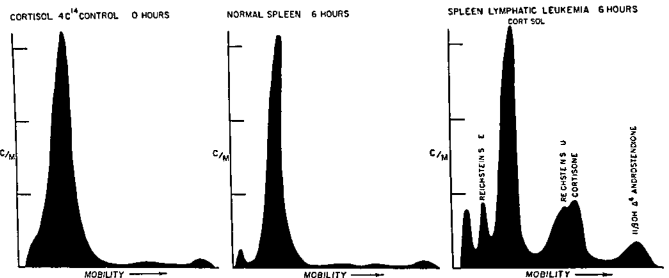



Fig. 1 Chromatograms showing peaks of radioactivity representing the metabolites of cortisol in the lymphatic leukemic tissue as compared with the recuperation of cortisol and normal spleen incubations

phatic leukemia These tumor cells catabolize cortisol but yield different compounds

The metabolic products of cortisol are formed in a cyclic fashion through the influence of the lymphatic leukemic cell (Fig 2) Cortisol goes to cortisone by a process of oxidation of the C 11 position This then through $\beta$ 20 reductase brings about a reduction in the 20 position producing a $\beta$ hydroxy compound Reichstein s substance U This product is then converted to Reichstein s substance E through reduction of the C 11 position It is most interesting that the cycle terminates in

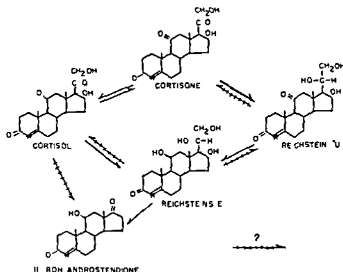



Fig 2 Cycle of transformations of cortisol by lymphatic leukemia cells

the production of a C 19 compound i e the whole side chain is removed and cortisol is eventually converted to 11 $\beta$ hydroxyandrostenedione This product cannot be reversed to any of the other catabolites so that the cycle here is interrupted and the C 19 compound is the final catabolic product

The reason we know this is a cycle is that incubates of 4 $C^{14}$ cortisol taken at hourly intervals show the progressive occurrence of the metabolites Furthermore the reversibility from cortisone to cortisol and from Reichstein s E to Reichstein s U has been established as well

The possible significance of these findings in relation to the etiology of leukemia can only be ascertained by future work It seems adequately

established however that cortisol is a normal regulatory hormone of lymphatic cell growth and differentiation It is possible that immature lymphocytes may also have the capacity to catabolize cortisol and in this way differentiation of the immature cell is inhibited In any case it seems warranted to hypothesize that a significant difference between normal maturing lymphocytes and lymphatic leukemic cells is that the hormone which influences normal lymphopoiesis is catabolized and converted to ineffective lymphocytolytic (tested in our laboratory) products by the leukemic cell In this way the leukemic lymphocyte maintains itself in a persistent immature state

REFERENCES

1 Dougherty T F *Physiol Revs* 32 379 (1952)
2 Dougherty T F *Recent Progr Hormone Research* 7 307 (1952)
3 Dougherty T F and White A *Am J Anat* 77 81 (1945)
4 Pfeiffer E F Sandritter W and Schoffling K *Klin Wochschr* 43 1023 (1952)
5 Santisteban G A and Dougherty T F *Endocrinology* 54 130 (1954)

## General Discussion

DR SALVATORE P LUCIA (San Francisco California) I should like to make a statement in regard to Dr Craddock's paper which I think is an unusually good one especially since it gives us some means of understanding the characteristic responses of the hematopoietic system and how it reacts to recovery from stress reactions induced in it

It reminds me of some very early experiments we did long before we could determine nuclear reactions of affected tissues I remember some of the peculiar leukopenias that were induced by marrow toxic agents especially amidopyrine and we observed then that if the marrow taps demonstrated a marked depression within the marrow the patients did not recover If the marrow were loaded with cells however even though there was no response to any of the so-called stimulating agents in the peripheral blood namely the pentose and hexose nucleotides those patients recovered Curiously the recovery began to be manifested along about the fourth day—the same type of reaction which Dr Craddock has observed in his animals I wonder if he has performed any experiments in his animals to demonstrate what was going on quantitatively or qualitatively in the marrow during these stress reactions after leukophoresis

DR ISRAEL DAVIDSOHN (Chicago Illinois) I too have a question for Dr Craddock He mentioned that there was no effect on the maturity of the white cells

appearing after the leukopheresis experiments in other words there was no shift to the left What I should like to know is whether there was any change in the granulocyte lymphocyte ratio

Another thing he mentioned was that apparently there was no change in the bone marrow Could it possibly be due to the fact that this was a rather short term experiment? What was the longest time that the animal were subjected to the leukopheresis?

DR E A MCCULLOCH (Toronto Canada) I should like to make a comment on the cultivation of leukocytes

In several laboratories including that of Dr Raymond Parker in Toronto long term cultures of various tissues of mesenchymal and epithelial origin have been observed to undergo sudden morphologic and physiologic change after which the cells multiply rapidly and may be washed centrifuged and subcultured with ease The same phenomenon occurs in cultures of leukemic leukocytes After 24 hours cultivation these cells have lost the distinctive features of the leukocytes of the body The nucleus occupies most of the cell and the cytoplasm is scanty and devoid of specific granules During the next 2 to 4 days the cells spread out on the glass and develop prominent undulating membranes The nucleus is central with a single nucleolus the cytoplasm is dense coarsely granular and stains blue with Wright s stain For – to 8 weeks these features remain although the cells increase in size During this period the population of the culture declines in spite of continued mitosis This decline may continue until all the cells are dead If continuous cultivation i to be achieved the cell must become altered in appearance and behavior The new cells which resemble Earle s L strain in appearance multiply vigorously and cells of the previously dominant type are no longer observed Once these altered cells have appeared the strain may be maintained indefinitely thus providing a plentiful supply of relatively uniform cells useful in many types of experiments However the altered cells cannot readily be identified with their ancestors in the body

There are two suggestions that I wish to make First I feel we should exercise great caution in applying ordinary hematological names to any cells that have been maintained in culture even for a short time Second experimental results obtained with cells in culture should not be used without reserve to explain the behavior of cells still present in the body This is especially important when long term culture has been achieved

DR EDWIN E OSGOOD (Portland Oregon) We have observed changes in cell over time but as you noticed we had almost every type that we saw later as early as 24 hours to 8 days in the culture We have interpreted some of these variations as acclimation to the medium and to the particular conditions of culture and we have noticed that if we change from human serum to horse serum there is a short period of adjustment

We would agree that certainly the tissue culture environment is not identical with the environment within the body but I do feel that studies on these cells may prove very useful in revealing more about what the metabolic requirements of cells are

We did notice that cells from the chronic leukemias tend to become more acute in culture and we regard this as a second mutation in its broadest sense leading to

a further shortening of the life span We have also noted that removal of the mature cell may be necessary in the culture of normal cells

There is so much left to learn that I hesitate to make any definitive interpretation of just why these cells are so difficult to grow for the first 6 months and so easy to grow thereafter

DR JØRGEN KIELER (Copenhagen Denmark) The transformation of the hemic cells into fibroblast or fibrocytic cells as far as morphology is concerned in tissue culture has been observed by many authors and it is of course a running discussion whether these cells are derived from the typical blood cells or whether they may be a specific cell type

I think you should be cautious not to base too much on the morphology of cells in vitro It is known that you may get quite different impressions of the type of cell you are working with when you are studying them under various conditions I think Dr Osgood has mentioned in one of his papers that fibroblast like cells when they are smeared may turn out to be real hemic cells

What type of cells they are—these cells which were just shown—I think we should leave for the future to decide

DR C G CRADDOCK JR (Fort Ord California) The fact that we do not observe much change grossly in the marrow agrees with the opinion that the amount of cells that we remove by leukopheresis even if we carry it out for a long period of time—and we have carried out such procedures for as long as 24 hours—is only a small fraction in terms of say 0 1 to 0 2% of the number of cells in the bone marrow

In other words what the marrow does when we leukopherese is merely to respond to the stress (if you want to call it that) by releasing cells that it has in storage The factor of increased production of cells in terms of new cell production and accelerated maturation and growth is a slower phenomenon and requires several days to occur

The only consistent changes which we noticed in the marrow of these normal dogs were some degree of myeloid hyperplasia in the marrow and left shift in the blood at the time 4 to 5 days after the procedure had been initiated

We feel that this indicates that there is a large reserve supply of leukocytes within the bone marrow and that even if production is totally ablated as by whole body irradiation there are relatively enormous numbers of these cells in the marrow which can come out into the peripheral blood and maintain the leukocyte numbers for some time unless there is also accelerated distribution of these cells to the tissues as there is in heavily irradiated animals

As stated in the paper the response to leukopheresis involves neutrophilic leukocytes only The lymphoid cells do not take part in the leukocytosis after the procedure

# 17

# The Influence of Cellular Fractions of Whole Blood on the Course of Acute Lymphocytic Leukemia A Preliminary Report

HOWARD R BIERMAN KEITH H KELLY PILAR A CENTENO and FAUNO L CORDES

Hospital for Tumors and Allied Diseases and Medical Research Institute City of Hope Medical Center Duarte California

The course of acute lymphocytic leukemia is often progressive and inexorably failing On frequent occasions however the clinical and hematological status will reach an apparent state of quiescence for variable periods In rare instances the leukemic state without apparent reason has been observed to undergo remission temporarily with disappearance of the clinical and hematological attributes so diagnostic of the leukemias The fact that a rapidly progressive disease can establish a state of equilibrium suggests that some restraining influences on the course of the leukemias may exist

Since the level of leukocytes in the peripheral blood in one sense reflects and perhaps regulates the hemostatic state (4) it was thought that the leukocytes themselves perhaps through their products might influence the physiological behavior of leukocytes and hematopoiesis in general

Leukopheresis in dogs is an efficient leukopoietic stimulant (3 7 8) This procedure requires the separation of leukocytes from large volumes of whole blood by centrifugation and the withholding of leukocytes and the reinfusion of the plasma red cells and platelets

The problem of leukopheresis in man is more formidable requiring the separation of leukocytes from 5 to 20 l of whole blood per hour as compared to 1 to 3 l per hour in the dog In the course of such separation large numbers of leukocytes and platelets are undoubtedly damaged and fragments of these leukocytes and platelets as well as the products thereof together with some intact and slightly damaged leuko

cytes are probably reinfused inadvertently with the red cell mass and plasma

The Cohn blood fractionator was originally devised to separate large volumes of plasma from whole blood (17) Its use as a device for rapid leukopheresis in man was undertaken with the full cognizance of its limitations The operation of the Cohn fractionator at flows of approximately 100 to 120 ml/min (roughly twice that recommended for optimal separation of leukocytes) and at 5000 rpm (3800 rpm is the recommended speed) results in disintegration of a large number of leukocytes (13) The separation of leukocytes from whole blood under these circumstances results in a cellular preparation which contains leukocytes platelets and various products of disintegration The infusion of these cellular preparations affords the opportunity to observe the effects of white cells plus cellular disintegration products These observations serve as a starting point in leukopheresis in man

## Methods

Eleven children with acute lymphocytic leukemia repeatedly received intravenous infusions of fresh cellular preparations of whole blood All diagnoses were proved by at least two bone marrow aspirations repeated observations of the peripheral blood the history physical examination clinical laboratory findings and the clinical course of the disease

All studies were conducted under strict hospital observation In all instances the patients were critically ill failing and usually with active hemorrhagic phenomena Antileukemic chemotherapeutic agents had been tried previously in 9 of the 11 patients Standard hematological procedures were employed as previously described (5) National Bureau of Standards certified hemocytometers and Trenner automatic filling pipets were used throughout Differential leukocyte counts were usually made by the supravital technique and confirmed later by fixed preparations with Wright's stain Microhematocrit determinations were employed in a majority of instances The remaining observations were by standard hematocrit procedures or colorimetric hemoglobin determinations

The cellular preparations of blood were obtained from compatible donors (ABO and Rh) and processed through the ADL Cohn blood fractionator The cellular preparations consisted largely of platelets and leukocytes and are specifically to be distinguished from what is classically known as the buffy coat Various factors of separation in the Cohn blood fractionator such as centrifugal force rate of separation tempera

ture and rate of flow of donor blood into the fractionator materially influence the composition of the cellular fraction

For clarity and simplicity of terminology the cellular fractions of blood derived from the Cohn fractionator were arbitrarily designated cellular preparations (CP) with numerical subscripts to denote the revolutions per minute which determined the centrifugal force during which the fractions were obtained (13) Thus $CP_5$ was that fraction of the cellular component of whole blood spun at 5000 r p m and obtained from that portion of the fractionator flow which followed immediately after the plasma was delivered and continued until the hematocrit approached approximately 10 to 15%

These cellular preparations as separated from whole blood contained both intact and fragmented leukocytes and products thereof There was no apparent significant platelet or erythrocyte disintegration The results of 100 cellular preparations indicate that $CP_5$ derived from one unit of blood was equivalent to 220 000 platelets/mm³ or a total of $22 \times 10^{9} \pm 10 \times 10^{9}$ platelets There were 11 000 WBC/mm³ or $1\ 1 \times 10 \pm 0\ 2 \times 10^{9}$ leukocytes There were probably at least an equal number of leukocytes which were fragmented during separation and would therefore not be reflected in the leukocyte count The average hematocrit was $12 \pm 5\%$ There was sufficient plasma to make a total volume of 60 to 100 ml

Separation of the cellular preparations was completed within an hour after the blood was drawn from the donor and administered usually within 1 to 4 hours after separation Cellular preparations from two or three compatible donors were often pooled to afford infusions of 120 to 300 ml

## Results

Crude cellular fractions of whole blood ($CP_5$) as described above were administered to 11 patients with acute lymphocytic leukemia in the active progressive phase (Table I)

In 5 patients profound but temporary clinical or hematological changes characterized by a precipitous drop in the circulating leukocyte level were recorded within 4 days (Table I Figs 1 and 2) Large fluctuations in the platelet count were observed and 7 to 14 days later the pretreatment diminished platelet number began to return toward normal levels (Fig 3) One to three weeks later the hematocrit was observed to rise Marked clinical improvement was noted characterized by a sudden sense of well being increased activity and loss of irritability and associated with a decrease in hepatosplenomegaly and marked decrease in cervical axillary and inguinal adenopathy

Bone marrow aspirates in 3 of the 5 patients became predominantly granulocytic from a previously completely lymphocytic myelogram (Fig 4) Immature lymphocytes were still detected in these marrows how

GRA ♂ 5 Acute Lymphocytic Leukemia

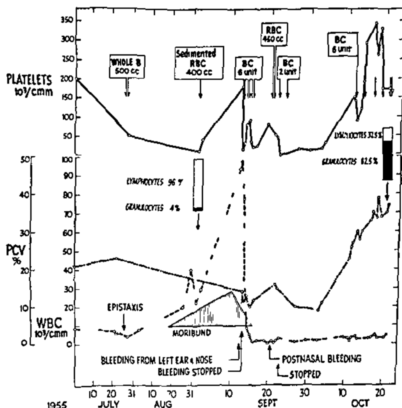



Fig 1 Clinical course of a 5 year old boy with acute lymphocytic leukemia Administration of cellular preparation on 9 13 (noted as BC on chart) primarily for bleeding when child was moribund with prompt control Subsequent administration of cellular preparation for recurrence of postnasal bleeding which also responded promptly to infusions of $CP_5$ derived from 2 units of whole blood Note the rise in hematocrit during the following month without any therapy other than cellular preparations also temporary rise in platelet level during the 2 weeks after administration of $CP_5$

ever although markedly reduced in percentage Increase in early erythropoietic cells was noted No destruction of the marrow elements was observed

Of the 6 remaining patients 3 exhibited slight clinical improvement

with an increase in circulating platelet and hematocrit levels but without marked alterations in the leukocyte number or differential In all these patients no significant change was observed in the bone marrow aspirates and only minor alterations in erythropoiesis were observed in

GRA # 5 Acute Lymphocytic Leukemia

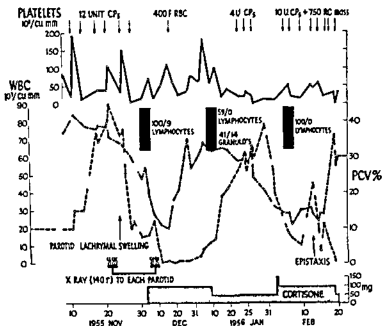



Fig 2 Subsequent course of GRA (5-year old boy with acute lymphocytic leukemia) illustrating the second fall of leukocyte number after reinstitution of infusions of $CP_5$ Bone marrow in the blocked area showing 100% lymphocytes of which nine were lymphoblasts Despite the administration of cortisone (100 mg/day) note the rising leukocyte level and falling hematocrit Bone marrow reverted from 59% lymphocytic and 41% granulocytic to 100% lymphocytic picture Despite administration of cellular preparation repeatedly and elevation of the hematocrit to 30 the child expired with a leukocyte level of 350/mm $^3$

these marrow aspirates Some decrease in lymphadenopathy and hepatosplenomegaly with slight clinical improvement was observed in 3 of these 6 patients In the remaining 3 patients no significant clinical or hematological changes were observed

No untoward reactions were observed with the repeated administration of $CP_5$ except in 2 patients who experienced mild urticaria toward

the end of a transfusion of the cell preparation which responded promptly and completely to antihistamine drug therapy

The clinical and hematological changes were temporary in all instances lasting from 2 to 8 weeks Eight of these 11 patients expired within 5 months after the initiation of CP infusions (Table I)

One child GRA who initially had exhibited dramatic improvement

REE ♀ 5 Leukemic Phase of Lymphosarcoma

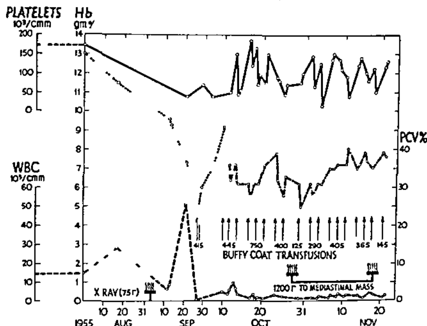



FIG 3 Chart of 5 year old girl with acute lymphocytic leukemia in conjunction with lymphosarcoma illustrating prompt fall in peripheral leukocyte level with elevated hemoglobin level after an infusion of $CP_6$ (listed on chart as buffy coat transfusion) from 4 units of whole blood Packed cell volume was substituted for hemoglobin determinations after September 10 Note fluctuating platelet level presumably related to the infusion of platelets

after the infusion of CP developed supraclavicular cervical adenopathy followed shortly thereafter by enlargement of the parotid and lacrimal areas (Fig 5) These proved exceedingly responsive to small doses of x irradiation (140 r to each parotid) This child eventually succumbed (5 months later) with an extensive femoral and iliac vein thrombosis despite all measures (Fig 6) The leukocyte count was at 350 mm³ Post mortem examination revealed only a few small lymph nodes without lymphocytic infiltration (Fig 7) Follicular atrophy in a 570-g

GRA ♂ 6 All — Marrow

7 28-55 MYEL — 1
LYMPHS — 99/50

10 21 55 PMN — 43
MYEL — 20
LYMPHS — 37/1
RBC — 18



a

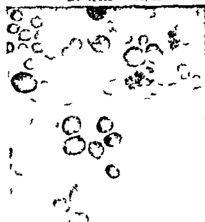


b

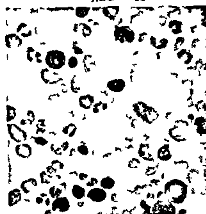
REE ♀ 6 All — Marrow

9-15-55 PMN — 2
MYEL — 3
LYMPHS — 95/41

12 _ 55 PMN — _4
MYEL — 21
LYMPHS — 55/10
RBC — 13



c



d

FIG 4 Comparative myelograms in 2 patients with acute lymphocytic leukemia *illustrating change to active granulopoiesis and erythropoiesis after repeated infusions* of $CP_5$ The code noting lymphs 99/50 designates 99% lymphocytes of which 50 were lymphoblasts

the end of a transfusion of the cell preparation which responded promptly and completely to antihistamine drug therapy

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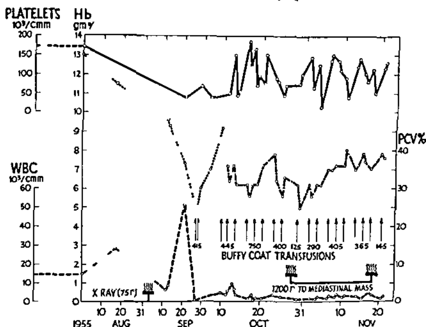



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*after* the infusion of $CP_5$ developed supraclavicular cervical adenopathy followed shortly thereafter by enlargement of the parotid and lacrimal areas (Fig 5) These proved exceedingly responsive to small doses of x irradiation (140 r to each parotid) This child eventually succumbed (5 months later) with an extensive femoral and iliac vein thrombosis despite all measures (Fig 6) *The leukocyte count was at 350 mm³* Post mortem examination revealed only a few small lymph nodes without lymphocytic infiltration (Fig 7) Follicular atrophy in a 570-g

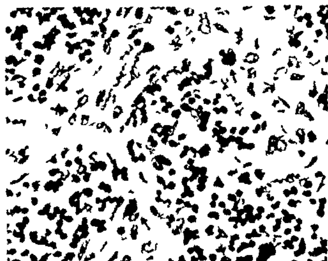


Fig 7 Mediastinal lymph node Leukemic lymphocytes are scarce accentuating the presence of reticulum cells Note lymphophagocytosis of reticulum cells in the medullary sinuses

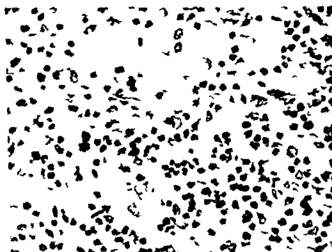


Fig 8 Spleen Extreme follicular atrophy Note the absence of follicular structure around the central artery with marked blood congestion and moderate hemosiderosis

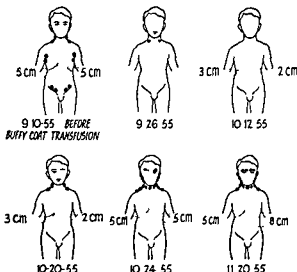



FIG 5 Schematic representation of enlargement of parotid cervical and lachrymal areas which *responded rapidly to 140 r to each parotid*
Note 11/22/55 to 12/1/55 Radiation Therapy to Parotid & Cervical Areas Responded Favorably Parotid & Cervical Glands Regressed But Liver & Spleen the Same



FIG 6 Left inguinal ligament about the femoral ring Formation of fibrin nets (thrombosis) in two veins and necrotic vessel walls (contributory cause of death)

**Discussion**

Well documented profound remissions termed "spontaneous" have been observed in the leukemic state (11) Wintrobe (18) reported a remission of several months duration in one case of acute leukemia after repeated transfusions He was unable to repeat this experience after renewed transfusions

Diamond and Luhby (10) reported an incidence of 9% spontaneous remissions in acute leukemia without any therapy whatsoever An additional 6% however had received blood transfusions (Diamond personal communication) Perhaps in these instances the term idiopathic remissions might be more suitable than "spontaneous" remissions



Fig 11 Kidney Diffuse infiltration of lymphocytes Note the presence of protein exudate in the glomeruli with edema of the renal papillae—the probable cause of death

The remissions obtained after exsanguination transfusions observed by Bessis and Bernard (1) and Croizat (9) may be evidence that the infusion of large volumes of blood in some manner causes profound ameliorative changes in certain of the leukemias

In two clearly defined instances temporary yet profound remissions in acute lymphocytic leukemia were observed in children who were cross transfused with large volumes of blood from hematologically normal donors (2) In still another instance in which an adult patient with chronic granulocytic leukemia was cross transfused with a child with

spleen (Fig 8) and lymphatic infiltration of a 1580 g liver limited to the portal regions was found (Fig 9) In contrast the kidneys were markedly enlarged to 280 and 300 g (Fig 10) and were grossly and microscopically infiltrated with lymphocytes (Fig 11)



Fig 9 Liver Marked infiltration of lymphocytes in the portal regions with proliferation of pseudo bile ducts



Fig 10 Kidneys bisected illustrate massive enlargement and infiltration with leukemic leukocytes

deed crude The leukocytes contain a myriad of complex bodies in both the cytoplasm and the nucleus each containing many chemical substances engaged in diverse reactions The transient nature of these changes and the fact that the changes could not be sustained despite repeated administration of these cellular preparations make many reservations essential

## Summary

Eleven patients with acute lymphocytic leukemia received a crude cellular preparation of whole blood $CP_5$ as obtained from normal compatible donors and processed by the Cohn blood fractionator Five of these patients exhibited a decrease in peripheral leukocyte count toward leukopenic levels and a gradual return of the platelet and hematocrit toward normal Marked general clinical improvement occurred in 3 of these 5 patients with a decrease in hepatosplenomegaly and lymphadenopathy and prompt cessation of hemorrhagic tendencies for 1 to 6 days Bone marrow aspirations in these 3 patients showed a change from a completely lymphocytic picture to one predominantly granulocytic All changes were temporary and could not be sustained by continued infusions of the cellular preparation ($CP_5$) The remaining 6 patients showed little or no significant changes

The data suggest that the cellular preparation ($CP_5$) of some donors as processed by the Cohn blood fractionator is capable of producing profound yet transient, hematological changes in some patients with acute lymphocytic leukemia The preliminary nature of these studies is emphasized

## REFERENCES

1 Bessis M and Bernard J *Bull mém soc med hop Paris* 63 871 (1947)
2 Bierman H R Byron R L Jr Kelly K H Dod K S and Black P M *Blood* 6 487 (1951)
3 Bierman H R Kelly K H Coblentz A Cordes F L and Leff D *J Clin Invest* 34 922 (1955)
4 Bierman H R Kelly K Cordes F L and Coblentz A *Blood* in press
5 Bierman H R Kelly K H Petrakis N L Cordes F L Foster M and Lose E A *Blood* 6 9[illegible]6 (1951)
6 Cooke J V *Trans Am Pediat Soc* 43 [illegible]6 (1931)
7 Craddock C G Jr Adams W S Perry S Skoog W A and Lawrence J S *J Lab Clin Med* 45 881 (1955)
8 Craddock C G Jr Perry S and Lawrence J S J Clin Invest 35 285 (1956)
9 Croizat R Revol L Viallet and Morel P *Le Sang* 18 474 (1947)
10 Diamond L K and Luhby L A *Am J Med* 10 238 (1951)
11 Forkner C E in Leukemia and Allied Disorders The Macmillan Company New York 1937

acute lymphocytic leukemia a remission for 3 months was induced in the child

In three instances the infusion of blood obtained from patients with chronic granulocytic leukemia and transfused into children with acute lymphocytic leukemia was followed by transient beneficial clinical and hematological changes (Bierman Kelly Cordes and Byron unpublished data) No similar effects were observed either in cross transfusion or simple transfusion of blood from patients with lymphocytic leukemia into patients with acute leukemia

These data suggest that temporary hematologic and clinical changes in the acute leukemias may be induced on occasion by large volumes of normal whole blood or by blood from patients with chronic granulocytic leukemia

Cooke (6) reported remissions of acute leukemia in children after the infusion of bone marrow extract On the other hand neither Forkner (12) nor Meyer *et al* (15) were able to produce any effect whatsoever by the administration of raw bone marrow lymph nodes spleen duodenum or extracts thereof

Previous work on infusion of cells and cell extracts by Cooke (6) Reznikoff (16) Marberg (14) and many others has been sufficiently suggestive as to make one anticipate that some unusual effects might be encountered after the infusion of crude cellular preparations and cell products To avoid the uncertain complications of solvents denaturation of cellular materials or inaccuracy of some types of extracts in the current study the crude cellular preparations were infused in the unaltered fresh state

Since many unusual hematological changes can be observed after infusion of crude cellular preparations one must also consider the possibility remote as it is that some of the hematological changes during chemotherapy with mustards x ray therapy the metabolic antagonists and the corticoids may in part be due to the products of destruction of the large numbers of leukocytes in addition to the direct effect of the chemical on the leukemic cell in its life history

Since it is known that the infusion of large numbers of erythrocytes into normal patients will be followed by a depression of erythropoiesis one may speculate that in a similar fashion the infusion of large numbers of leukocytes might depress leukocyte production In leukopheresis the removal of large numbers of leukocytes is followed by apparent stimulation of leukopoiesis in the marrow

It should be stressed that these are merely preliminary observations and must be interpreted accordingly The cellular preparations are in

# 18

# The Leukocytic Resistance Test

E. STORTI

Institute of Clinical Pathology of the University of Modena Italy

White blood cells placed in a hypotonic salt solution (0 20%) in time undergo lysis in ever increasing numbers But the time it takes for this to occur varies considerably depending on whether polymorphs or lymphocytes or other blood cells are treated Therefore it appears that diverse types of blood cells have different degree of resistance to the lytic action of the hypotonic solution

From the preceding constatation was born the concept of *leukocytic* resistance (L R ) which we formulated in 1951 this concept does not appear in the hematological literature which is surprising when one considers how fruitful was the analogous concept of erythrocytic resistance

To evaluate L R we proceed thus A determination is made every ½ hour for 3 hours of the percentage of the integral remaining white blood cells the polymorphs and lymphocytes being counted separately Thus we obtain two curves which correspond to the resistance of the white blood cells to the lytic action of the hypotonic solution

Figure 1 represents resistance curves of polymorphonuclear and mononuclear white blood cells in normal subjects After 3 hours in the solution 80% of the polymorphs but only 60% of the mononuclear cells are destroyed indicating the greatly different fragility of the two cellular types to the hypotonic solution These curves represent the average values computed statistically for 200 normal subjects We have also found that (*1*) the L R curves are constant in normal subjects within a constant variability band as shown by statistical calculations and (*2*) the curves present modifications of different direction and intensity depending on various physiologic and pathologic conditions

12 Forkner C E *Chinese Med J* **52** 1 (1937)
13 Kelly K H Bierman H R Cordes F L and Watson H *Federation Proc* **15** 445 (1956)
14 Marberg C M and Wiles H O *Arch Internal Med* **61** 408 (1938)
15 Meyer O O Middleton W S and Thewlis E W *Folia Haematol* **53** 166 (1935)
16 Reznikoff P *J Clin Invest* **9** 381 (1930)
17 Tullis J L *Blood* 7 891 (1952)
18 Wintrobe M M *in* Clinical Hematology p 713 Lea & Febiger Philadelphia 1946

between the single counts the pipet must be kept at normal room temperature (20 to 25 )

From the leukocytic figures resulting from these counts the percentages of decrease of white blood cells in respect to the initial count are obtained which when placed on the Cartesian diagram allow us to construct leukocytic resistance curves for the mono and polynuclear cells (see Fig 1)

The single counts are carried out with a Buerker chamber and microscopic observation by darkfield contrast and direct illumination For these counts recognition of the morphological characteristics of the leukocytes is aided by staining the cells lightly with Giemsa This staining is obtained by repeating the following procedure for each count

1 A tiny drop of Giemsa (about the size of a pinhead) is placed just in front of the Buerker cover slip

2 A drop of the pipet s contents is then allowed to fall on the drop of Giemsa the two drops are mixed in a slow rotatory motion with the tip of the pipet

3 Still with the tip of the pipet the liquid thus mixed (lightly colored in blue) is placed in contact with the margin of the Buerker cover slip

In such a way we obtain a homogeneous coloring of the cellular suspension and then through capillary action the Buerker counting chamber becomes filled up at this point the white blood cells are counted

Comparative determinations have shown that with this method of staining the concentration of the surrounding liquid is not sensitively modified and the results are not altered

The most delicate point of the entire determination is in being able to distinguish altered leukocytes from intact ones To avoid any mistaken ideas we think it is useful to show in Table I the morphological characteristics of the leukocytes which we judge to be intact and those which we hold to be altered and which (in the counts after the initial one) are not counted

When one intends to use an index of leukocytic resistance especially for comparative purposes we propose as an index the area of the surface enclosed between the time axis the ordinates of the extreme points of the curve and the curve itself

Such an area is deduced by means of the following formula

$$\text{Resistance index} = \int_0^4 y\, dx$$

$y$ being the interpolative function and $x$ being the time the unity of which when measured graphically has been set at 30 minutes

**Method for Determining Leukocytic Resistance**[1]

The necessary materials include a white blood cell counting pipet (preferably with a wide ampoule) a Buerker counting chamber a binocular microscope (a No 40 objective and a No 8 ocular 320×) NaCl solution exactly titrated at 0 20% and pure Giemsa (filtered a number of times until it is free of granules and precipitates)

Blood obtained from the fingertip is drawn into the counting pipet up to marker 1 and immediately after hypotonic NaCl solution is drawn

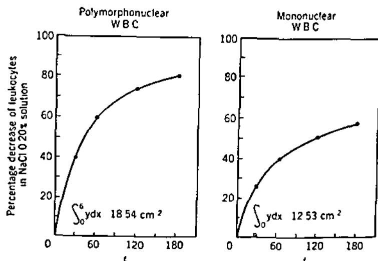



FIG 1 Resistance curves of polymorphonuclear and mononuclear white blood cells (average for 200 normal subjects) Resistance index $= \int_0^6 y\, dx$

up to marker 11 The pipet is agitated ten to fifteen times as is done with ordinary globular numerations and the first leukocyte count is immediately carried out The mononuclear ones are counted separately from the polynuclear ones however and both the intact and the altered elements (if there be any present) are computed Other counts are carried out with the remaining liquid in the pipet after 30 60 120 and 180 minutes only the intact elements are computed however During the intervals

[1] About two years after publication of our method (*Progresso Medico* September 1952) Richards and Richards presented to the International Congress of Clinical Pathologists in Washington (September 1954) their method of determination of leukocytic resistance to hypotonic solutions Their method differs from ours in technical details of execution and also in the fact that it does not differentiate between mono- and polynuclear cells

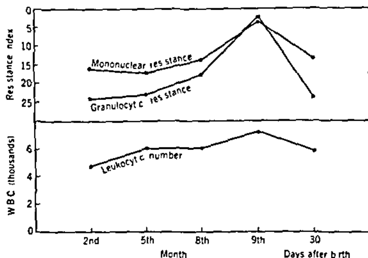



FIG 2 Leukocytic resistance in pregnancy



FIG 3 The effect of estrogens on leukocytic resistance

**Probable Significance of Leukocytic Resistance**

Our studies of the past few years made under the most diverse conditions lead us to believe that L R reflects an aspect of the biological condition of the white blood cells probably related to the condition of its membrane or its enzymatic or biochemical makeup it is not improbable that L R constitutes also an indication of the condition of the leukopoietic marrow

TABLE I

| | Mononuclear | Granulocytic |
|---|---|---|
| Intact leucocytes | | |
| Altered leucocytes | 1 2 3 4 5 | 6 7 8 9 / 10 11 12 |

1 6 8 11 12 = rupture of the membrane with loss of a fragment of the cytoplasm
2 4 = shriveling of the cytoplasm
3 = global alteration of the morphology of the cell
5 7 10 9 = Nucleus at the periphery of the cell and about to pass out of it

This presentation refers the variations in L R in the following conditions (*1*) physiological conditions such as exist during pregnancy or after prolonged administration of estrogens (*2*) grave marrow alterations e g benzol myelopathy (*3*) leukemias and (*4*) conditions due to the action of antimitotics

Leukocytic Resistance in Physiologic Conditions

Up to now we have found in normal subjects the most marked modifications of L R during pregnancy and after prolonged administration of estrogens In Fig 2 is shown the great increase in L R especially at the ninth month in a healthy pregnant woman and its decrease about *1 month after* childbirth Note the parallel increase *in* the number of white blood cells reaching the maximum also at the ninth month This behavior we believe is due principally to the high estrogen content which is found at term of pregnancy

In fact in normal subjects after estrogen administration we note a corresponding increase in number and resistance of white blood cells (Fig 3) We shall see how this action of estrogen on L R can lessen the intensity of the decrease in white blood cells due to antimitotics

blasts of the acute lymphatic leukemias are much less resistant than lymphocytes of normal blood

There is also a great difference between the resistance of the diverse types of myeloid leukemia cells In fact in Fig 7 we see that the re

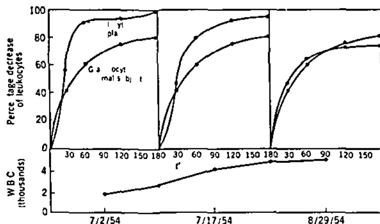



Fig 5 Marrow aplasia in recovery

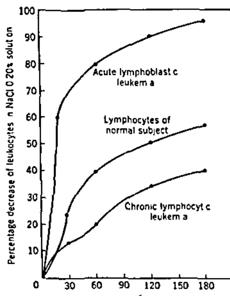



Fig 6 Lymphatic leukemia (ten cases examined)

Leukocytic Resistance in Marrow Aplasia

In cases of grave marrow aplasia L R of the granulocytes has always been found to be greatly decreased compared to that of granulocytes of normal blood This phenomenon is clearly shown in Fig 4 for a case of marrow aplasia due to benzol In cases of aplasia that are responding

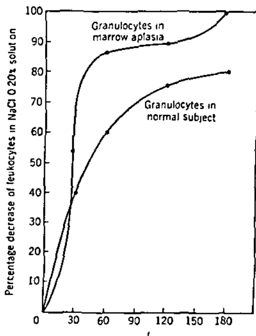



Fig 4 Modifications of L R in marrow aplasia

favorably it is interesting to note the progressive change of L R toward normal Figure 5 relative to the preceding case (benzol) shows *the parallel increase in the resistance of white blood cells and in their* number

Leukocytic Resistance in the Leukemias

The L R varies considerably depending on the cytological type of leukemia We shall consider first the chronic and acute lymphatic leukemias

Figure 6 demonstrates clearly that the lymphocytes of chronic lymphatic leukemia are more resistant to the hypotonic solution than lymphocytes of normal blood even though the latter are not distinguishable morphologically from the former On the other hand the lympho

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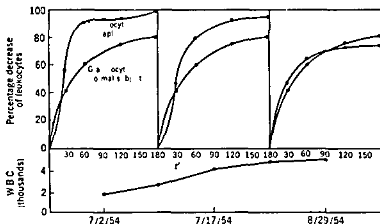



FIG 5 Marrow aplasia in recovery

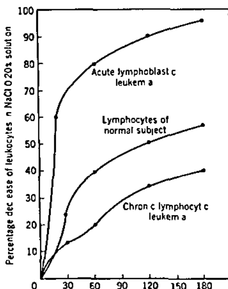



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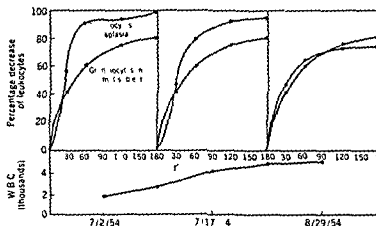



Fig 5 Marrow aplasia in recovery

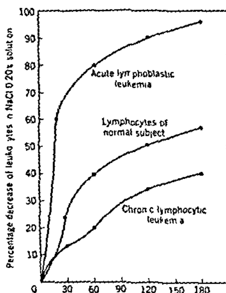



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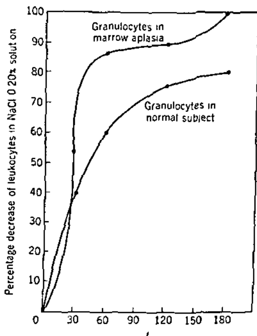



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Figure 8 shows that the hemocytoblasts in acute hemocytoblastic leukemia also have a resistance much higher than normal granulocytes and

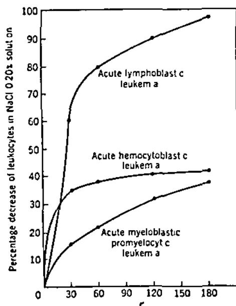



FIG 9 Acute leukemias (twenty cases examined)

lymphocytes but very close to that of the myeloblasts and promyelocytes of acute myeloid leukemia

In Fig 9 is shown the different degrees of resistance of the cells predominating in the blood of the principal types of acute leukemias with the lymphoblasts very fragile the myeloblasts and promyelocytes very resistant and the hemocytoblasts with intermediate resistance

We have also differentiated the acute myeloid from acute lymphoid leukemias on the basis of the histochemical characteristics of the cells

The above data indicate the following (1) Each of the principal types of leukemic cells has a resistance to hypotonic solution which is characteristic and constant (2) It is possible to differentiate the prin

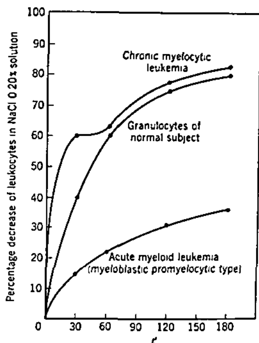



Fig 7 Myeloid leukemia (fifteen cases examined)

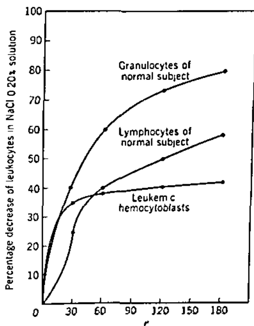



Fig 8 Hemocytoblastic leukemia (five cases examined)

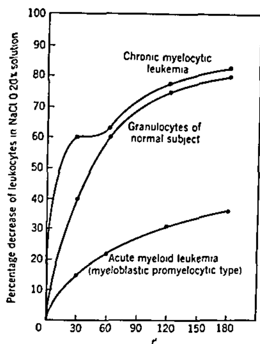



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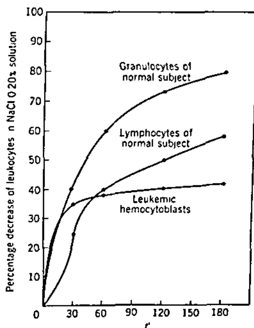



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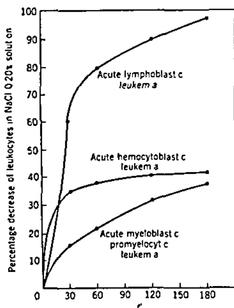



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cipal types of leukemia especially the acute forms the cells of which often are rather difficult to distinguish on the basis of the resistance of their blood cells

LEUKOCYTIC RESISTANCE DUE TO ANTIMITOTICS

*Hodgkins Disease* As I stated at the International Hematology Congress of 1954 (Paris) almost all common antimitotics effect a decrease in the resistance of white blood cells This is verified with almost absolute regularity if the antimitotic is administered to subjects with nor

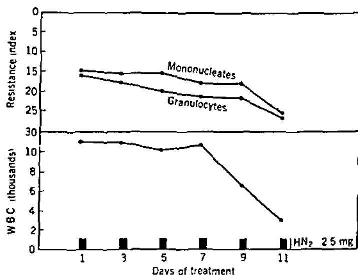



FIG 10 Decrease in L R in a case of Hodgkins disease treated with HN2

mal leukopoiesis Furthermore such a decrease in most cases precedes the decrease in number of white blood cells

Figure 10 shows a progressive decrease of L R in a case of Hodgkins disease treated with HN2 This decrease is already evident when the number of white blood cells are still more or less unchanged Therefore we see that the L R test can be utilized in treating cases with antimitotics with the purpose of avoiding dangerous leukopenias

*Leukemias Treated with Antimitotics* In the majority of leukemia cases (not however in all) the common antimitotics effect a decrease in the resistance of leukemic cells to the hypotonic solution as has been shown in the granulocytes and lymphocytes in Hodgkins disease In Figs 11 and 12 we have a few examples

Another noteworthy fact is that the steroid hormones whether of the

cortisone hydrocortisone prednisone or prednisolone type in standard doses do not cause a decrease in L R

Figure 13 shows that in the same case of chronic lymphoid leukemia a treatment with prednisone did not result in a decrease either in the number of white blood cells or in their resistance although a later treat ment with triethylene melamine provoked a rapid and parallel decrease

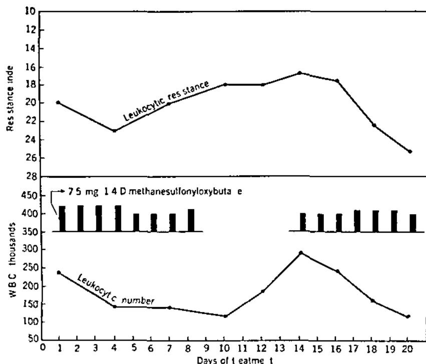



FIG 11 Chronic myeloid leukemia treated with d methanesulfonyloxybutane (Myleran)

in number and resistance In another case prednisone did not modify the resistance of the leukemic cells although it caused a decrease in their number

The fact that unlike the common antimitotics the steroids do not affect L R constitutes another element which leads us to suppose that these two types of medicaments act on leukemic cells by entirely dif ferent mechanisms

Figure 14 demonstrates however that in some cases of leukemia the antimitotic produces a decrease in the number of white blood cells and satisfactory clinical results without a corresponding decrease in resist ance of the leukemic cells Such behavior has never been seen by us in patients with normal leukopoiesis e g in Hodgkin s disease

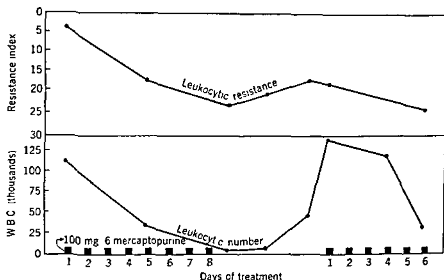



Fig 12 Acute hemocytoblastic leukemia treated with 6-mercaptopurine The change in the number of leukemic cells caused by the treatment corresponds to the change in the resistance of these cells to the hypotonic solution

## Leukocytic Resistance and Hypotheses on the Mechanism of Leukopenia Due to Antimitotics

We have shown the following to be true (*1*) In conditions of normal leukopoiesis the decrease in the number of white blood cells due to anti mitotics *is regularly associated with but preceded by parallel decrease* in their resistance This has been verified in the leukemias to some degree (*2*) However precocious and intense is the decrease in the re sistance of the white blood cells so much more intense and precocious is the decrease in their number (*3*) In cases of benzene marrow aplasia the resistance of the white blood *cells* is notably decreased

We further maintain that many antimitotics cause leukopenia through a double mechanism (*1*) at the center inhibiting the proliferation (the *known* cytostatic action of such substances) (*2*) at the periphery de creasing the resistance of the white blood cells thereby causing an in crease of the peripheral leukolysis

An extensive and very rapid decrease in the number of white blood cells which sometimes appears during antimitotic therapy is perhaps explained by recognizing the existence of this second mechanism For the moment however we can present only an hypothesis which necessarily must be confirmed by observations on a vast number of cases

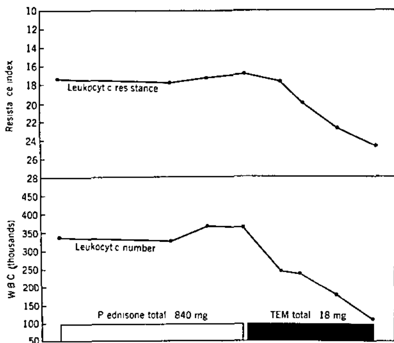



Fig 13 Chronic lymphoid leukemia treated first with prednisone and then with TEM

The above mentioned pathogenetic interpretation has also a practical value in as much as it gives us an insight into the possibility of lessening the decrease in white blood cells due to antimitotics thus causing an increase in resistance

In fact at the recent Congress of Hematology (Geneva May 1955) my co worker Pederzini and I showed that administering diethyldioxystilbene dipropionate (synthetic estrogen) during the course of antimitotic therapy in many cases reduces the intensity of decrease in white blood cells caused by antimitotic therapy The possibility of employing the estrogen for such a purpose came to us after our observations of the

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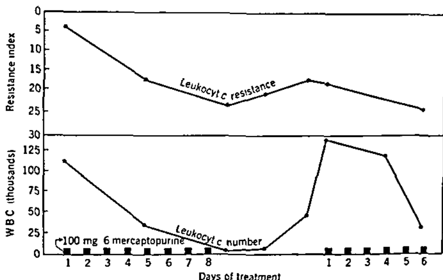



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but not in another analogous case would seem also to verify the great variability in the response of leukemias to antimitotic therapy

From a practical point of view it is evident that when a decrease in L R appears during antimitotic therapy it is justifiable to expect an imminent fall in the number of white blood cells Thus we have established the usefulness of employing the L R test in the practical conduct of therapy of leukemias with antimitotics

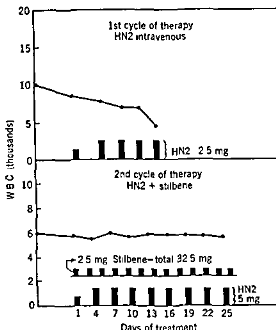



Fig 15 Case of Hodgkin s disease treated first with HN2 and then with HN2 + *stilbene*

## Conclusion

On the basis of the few constatations set forth above it would seem that we can affirm that the resistance of the leukocytes to hypotonic saline solution can furnish interesting information of a qualitative order on the single leukocyte and probably also on the condition of the leukopoietic tissue The leukocytic resistance test therefore constitutes an exploration of the physiology and physiopathology of the leukocytes It takes its place alongside other tests already known to us but it has the advantage of being very easy to perform and therefore extremely ap

notable increase in L R which manifests itself at the ninth month of pregnancy

Figure 15 relative to a case of Hodgkin s disease shows that in the first cycle of therapy after 22 5 mg of HN2 the white blood cell count fell from 10 000 to 5000 *in the second cycle after stilbene was administered* at the same time as the antimitotic the count fell from 6000 only to 5500 in spite of the fact that 42 5 mg of HN2 was administered

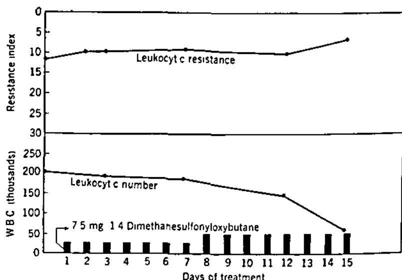



Fig 14 Chronic myeloid leukemia treated with dimethanesulfonyloxybutane

Let us now return to the argument that leukopenia can be caused by antimitotics I have already said that among the cases studied by us there are a few in which the antimitotic brought about a notable decrease in the number of white blood cells but no decrease in their resistance In regard to these cases it would seem that we can conclude that the antimitotic brought about only a cytostatic effect and not a cytolytic effect

Now then we have seen both effects produced by the same antimitotic in different cases of leukemia which were of similar type

We have never observed this in cases of normal leukopoiesis or in conditions not of leukemic type since as has already been said in such cases the antimitotics have regularly caused a decrease of L R although in a very different measure

The fact (as indicated by the pattern of L R ) that the same antimitotic can cause destruction of leukemic cells in one case of leukemia

This apparatus was designed for a flow rate always of less than 50 ml/min and a gravitational force of about 100 × g If one wishes the apparatus can yield pure platelets with essentially no red cells pure red cells with essentially no platelets and mixed populations of white cells in a proportion of about four white cells to one red cell

DR SERGIO DECARVALHO (Cleveland Ohio) Concerning the work of Dr Bierman I should like to add a brief comment We too are using fractions from human leukemic cells but in an entirely different way Cytoplasmic fractions of acute leukemic cells are inoculated in standard cultures of human amniotic cells After several subinoculations the culture fluid is injected into rabbits The immune antiserum obtained shows a selective lytic activity for the original leukemic cells

In one adult case of acute leukemia after the use of this serum the leukemic cells disappeared both from the blood and from the bone marrow and a remission either spontaneous or induced followed this administration for about one and a half years

Now if I may go back to the work of Dr Kieler I am very anxious to ask him a question The results of Dr Kieler's work especially those obtained on acute leukemic cells do not seem to me to be comparable with the results obtained by Dr Warburg lately on individual malignant cells Since Dr Warburg in a recent publication was so positive in affirming that very high fermentation and very little respiration are characteristics of malignant cells independently of oxygenation I should like to ask Dr Kieler how he feels about this

DR JØRGEN KIELER (Copenhagen Denmark) I am sorry that the paper that was just referred to by Dr Warburg has not arrived at my laboratory yet so I am not familiar with it although I have had a chance here to have just a glimpse at it

Of course the results we have obtained are in some respects contradictory to Warburg's hypothesis I do not think however that it is possible on the basis of our observations to make a general statement as to the metabolism of cancer cells On the other hand since cancer cells in all probability have a high energy metabolism I am satisfied with the fact that malignant cells at least in the case of leukemia may obtain their energy through respiration which is a much more efficient way of getting energy than through glycolysis Other malignant cells should be examined with regard to the influence of oxygen tension on their respiration The results of such experiments may lead to modifications of Warburg's general theory in which the glycolytic energy metabolism plays such an important role

DR SVEN MOESCHLIN (Soluthurn Switzerland) I want to felicitate Dr Craddock on the wonderful work he did on the increased destruction of the leukocytes in the periphery I think this work will be of great importance for the future because it proves in a very nice manner that the increased destruction of leukocytes is probably one of the main factors in granulocytopenia and leukocytosis

On the other hand, I think the work of Storti is also very interesting and I am sorry that he could not find a difference in chronic leukemia from normal leukocytes We still find it impossible to correlate in leukemoid reactions various differences because I presume they have also the same normal response And so the whole problem of osteomyelosclerosis which would be very interesting to study will probably not be solved by this method

plicable in current practice—e g for a more precise observation of leukopoiesis during the course of antimitotic therapy or perhaps as a means of determining the degree and mechanism of other antimitotics on the white blood cells

REFERENCES

1 Bellesia L and Lusvarghi E *Progr med* 12 40 (1956)
2 Bussi L and Boesi G *Med intern* 12 433 (1955)
3 Curtarelli G and Bianchi P G *Boll soc ital ematol* 2 352 (1954)
4 Parrella M *Arch fisiol (Naples)* 8 592 (1953)
5 Pederzini A Bellesia L and Lusvarghi E *Min med* 1 49 (1955)
6 Pederzini A Bellesia L and Lusvarghi E *Progr med* 8 566 (1952)
7 Pederzini A Bellesia L and Lusvarghi E *Progr med* 11 490 (1955)
8 Pederzini A Bellesia L and Lusvarghi E *Progr med* 11 745 (1955)
9 Pederzini A Colombini M Bellesia L and Lusvarghi E *Progr med* 9 726 (1953)
10 Pizzetti F Clerici E and Di Pietro S *Tumori* 5 528 (1954)
11 Richards H G H and Richards D L *Intern Congr Clin Pathol* Washington September 1954
12 Storti E *Rev hematol* 10 492 (1955)
13 Storti E and Pederzini A *Progr med* 8 563 (1952)
14 Storti E and Pederzini A *Giorn ital chemioter* 1 169 (1954)
15 Storti E and Pederzini A *Schweiz med Wochschr* 38/39 949 (1955)
16 Storti E and Pederzini A *Acta Med Scand* 154 417 (1956)
17 Storti E Pederzini A and Mauri C *Rass med* 32 17 (1955)
18 Storti E Pederzini A and Mauri C *Rass med* 32 11 (1955)
19 Zannoni D and Pane G *Progr med* 9 653 (1953)

## General Discussion

DR JAMES L TULLIS (Jamaica Plain Massachusetts) I should like to comment on Dr Bierman s paper I have no remarks to make on the use to which he has put the Cohn fractionator or indeed on the interpretations of his clinical results I feel however that I must rise in defense of what I believe is a totally erroneous interpretation that only *crude* cell fractions can be obtained from this machine

I did not invent the apparatus but as one who has worked five years in its development I have the interest of a stepfather and at least some impression of what can be done with it

This apparatus was not devised for leukopheresis experiments and as a result of this it cannot be interpreted as being immediately applicable to such techniques If one uses the flow rates of 120 ml/min and centrifugal force of 5000 r p m as Dr Bierman has done and has shown on his graph it would be impossible to obtain anything but lysed white cells

tions or cellular preparations from fresh whole blood containing primarily platelets and white cells might have some of this material that might correct the defect in the bleeding leukemic

Also it is apparent that the bleeding defect in the leukemias is probably a multiple entity Platelet infusions although temporarily beneficial in controlling the bleeding phenomena will not protect for prolonged periods We felt that perhaps by adding both the white cell and the platelet we might be adding more than one or two of the components that may be deficient

Indeed clinically that seems to have worked out In 31 of 50 recorded instances of active bleeding in 22 leukemic patients the hemorrhages have been stopped for at least 1 to 6 days almost immediately after the infusion of $CP_5$ In 19 instances although bleeding was halted in most it did not persist for 24 hours

In answer to Dr Lucia in most instances the platel t level did go up slightly but not consistently The number of platelets that were infused was often insufficient for a sustained rise Steffanini and Dameshek showed that one has to give approximately 4 billion platelets per pound of body weight to be able to obtain any significant rise

Dr Edoardo Storti (Modena Italy) In reply to Dr Moeschlin s remarks it is true that the leukocytic resistance of the leukocytes in chronic myelo d leukemia is very similar to that of the normal granulocytes It is necessary however to note that this behavior is characteristic of myeloid leukemia in its chronic form and of cells that are very mature As soon as a case of chronic myeloid leukemia begins to show an unfavorable course and the percentage of myelocytes and promyelocytes in the peripheral blood increases however the leukocytic resistance also increases as compared to the normal granulocytes

Actually this modification of the leukocytic resistance appears quite early and we use this phenomenon as prognostic criteria

Dr A S Gordon (New York New York) The marrow picture in the rat would support strongly Craddock s contention that there is a tremendous reserve of leukocytes I have just made a rapid calculation based on our own data which indicates that the mature neutrophils within rat marrow outnumber those in the blood circulat on by a factor of 80 This would imply that there is an 80 fold reserve of neutroph ls that could be mobilized rapidly to replace the total population in the circulation

With regard to Dr Storti s observations we had some brief exper ence with leukocytic as well as erythrocytic resistance some years ago when I worked with Dr Eric Ponder We found that the resistance of the cells varied depend ng on the type of lysin used For example the resistance of the blood elements toward such lytic agents as hypotonic saline and sodium taurocholate was of a different order from that shown toward saponin

I should like to suggest that other lysins in addition to hypotonic sal e be used in the Storti d agnostic test This approach might yield additional valuable informa tion regard ng the nature of the leukocytic surface in various disease states

DR SALVATORE P LUCIA (San Francisco California) I should like to say some thing in partial defense of the experiment of Dr Bierman I should like to state that he was able to get a remission of some significance in these children Whether it is a cause and effect reaction is difficult to ascertain but at least those who have treated leukemia in infants realize that if this is true without any reference to the researcher it is significant" What its ultimate meaning is is another matter en tirely

I should like to know this First regardless of the techniques of the preparation of this material which I think was pretty heroic stuff to deal with did these children react with a fever reaction or a severe systemic response after the administration of the material? Second was there any evidence of purpura in these youngsters before the administration of the material and was it influenced since we saw that there had been remissions in the peripheral blood in the bone marrow and in the hemolymphopoietic organs as well? Lastly did you see as I saw a rather startling reciprocal relationship between the decrease in the white blood cell count and the maintenance or increase in the platelet count?

DR H R BIERMAN (Duarte California) First of all I want to pay due respect to Dr Tullis and his group because they taught us how to use the fractionator The Cohn Tullis fractionator was not designed originally for leukopheresis and as with many devices applications other than for the specified purpose often sacrifice one advantage for another

The Cohn fractionator can give good platelet rich plasma preparations even at 5000 r p m As I tried to make clear in the paper we were not primarily interested in obtaining completely intact leukocytes but rather in rapid separation and infusion to prevent the loss of possible labile components of the leukocytes Smears of leukocyte preparations obtained by different processes usually reveal much cellular disintegration despite all precautions We had previously studied the infusion of intact leukocytes and other cells by cross transfusion In this study we estimated about half of the cells were in some stage of disintegration

At 4 200 r p m or slower rates of flow one can get better cellular preparations at higher flow rates the purity of the preparation is sacrificed for fresher material

In reply to Dr DeCarvalho I do not believe at any time in this discussion that we talked about these studies as treatment These are strictly preliminary observa tions to determine what happens when large numbers of fresh leukocytes and platelets are infused

I believe it is very significant that this material despite its continued adminis tration at 2 to 4 day intervals was unable to sustain these effects The observation that stored cellular preparation has shown no activity suggests that some of the factors are readily labile In answer to Dr Lucia s question Initially we employed the cellular preparations because of its possible antihemorrhagic potency It is well known that normal fresh whole blood or polycythemic fresh whole blood is very efficient in controlling the generalized bleeding in leukemias whereas bank blood is markedly less efficient The major difference between fresh and stored blood aside from postulating or speculating on some unusual plasma constituent would be plate lets and white cells since most bank blood is administered at least 6 or 10 days after drawing and most of the platelets and white cells would have disintegrated by that time

Therefore it appeared reasonable to assume that perhaps crude cellular prepara

# Part VI

# Metabolism of Amino and Nucleic Acids in the Leukemias

Chairman
L. J. Witts
Nuffield Department of Medicine
University of Oxford
Oxford, England

# 19

# Some Aspects of Amino Acid Metabolism in Leukemia

HARRY A WAISMAN
Department of Pediatrics Medical School
University of Wisconsin Madison Wisconsin

## Introduction

One of the dominant roles in cellular metabolism is held by amino acids and by certain key proteins Their importance in the formation of nucleoprotein enzymes and other cellular content is well established Relatively little is known however about amino acid distribution in plasma or in the formed elements of blood and even less data are at hand on the changes that occur in these substances during the course of disease or after therapy

For several years it has been possible to pursue certain phases of amino acid metabolism in human leukemia and in mouse and rat leukemia This report will deal mainly with amino acid metabolism in various human leukemias and mouse leukemia As quantitative methods are refined and as more information is obtained it may be possible to relate the function of some amino acids more directly to specific enzyme action in addition to the more traditional structural role that is usually assigned to those building blocks" that make up proteins

## Free Amino Acids in Plasma

For many years it has been an accepted fact that the plasma amino acids remain relatively constant except for transitory increases after the intake of food Prior to the development of more specific methods for the quantitative determination of individual amino acids earlier investigators found that amino acid nitrogen was higher in patients with nephritis and leukemia (18) Becher and Herrmann (5) found elevated amino nitrogen values in the leukemic patients they studied This increase in blood amino nitrogen was related to the elevated basal metabolic rate as first noted by Grafe (11) in 1911 and later commented on by

values than normal children of the same age The values are statisti cally significant Normal adults have the same tyrosine values as normal children and it is of interest that patients with chronic granulocytic leukemia and chronic lymphocytic leukemia as well as cancer have values that are distinctly higher than those in normal individuals (Table III) It is worth nothing that despite therapy or temporary remission of the disease the amino acid levels were unchanged This work confirms

TABLE II
PLASMA TYROSINE LEVELS IN NORMAL AND LEUKEMIC CHILDREN

| Classification | Number of patients | Mg/100 mL plasma | | P | |
|---|---|---|---|---|---|
| | | Mean | S D | | |
| Normal | 11 | 1 13 | 0 11 | —— | |
| Leukemic | 11 | 1 87 | 0 37 | < 0 01 | 0 07 |
| Treated leukemic | 11 | 1 62 | 0.23 | < 0 01 | |

TABLE III
PLASMA TYROSINE LEVELS IN NORMAL AND LEUKEMIC ADULTS

| Classification | Number of patients | Mg/100 ml plasma | | Range | P |
|---|---|---|---|---|---|
| | | Mean | S D | | |
| Normal | 16 | 1 10 | 0 14 | 0 8–1 3 | |
| Untreated chronic lymphatic | 8 | 1 [illegible]4 | 0.26 | 1 0–1 63 | 0 0[illegible] |
| Treated chronic lymphatic | 15 | 1 40 | 0 44 | 1 0–2.5 | < 0 01 |
| Untreated chronic granulocytic | 12 | 1 47 | 0 [illegible]0 | 1 2–1 9 | < 0 01 |
| Treated chronic granulocytic | 7 | 1 76 | 0 67 | 1 1–3 1 | < 0 01 |

microbiological determinations and further supports the thesis that both phenylalanine and tyrosine interrelated as they are in metabolism are apparently functioning in the same direction in the patient with leukemia

We had noted earlier (28) that hyperpigmentation of the skin oc curred in children receiving the folic acid antagonist aminopterin for long periods of time The darker colored skin appeared prominently over the ankles back and fingers in some white children Confirmation of the clinical impression of hyperpigmentation was obtained when skin from cases at autopsy demonstrated the presence of melanoblasts (14) in

Murphy *et al* (17) Further work by Riddle and Sturgis (20) and by Krantz and Riddle (12) also demonstrated the elevated basal metabolic rate Baldridge and Barer (2) made the observations that protein catabolism was directly related to the increase in oxygen consumption and therefore responsible for the increased basal metabolic rate No further effort was made to explain this phenomenon and little or no information has been available on the disordered amino acid metabolism that undoubtedly exists in this group of diseases

To define more clearly one aspect of amino acid metabolism free amino acids in the plasma of normal and leukemic patients were determined A micro adaptation of an established microbiological procedure enabled us to use 3 to 5 ml of plasma in order to analyze for twelve of the essential amino acids (27) It was possible to use a total incubating volume of 0 2 ml and to neutralize the acid produced from bacterial growth by means of an electronic titrimeter and concentric glass electrode (7) As will be seen in Table I significantly higher values

TABLE I
FREE AMINO ACIDS IN PLASMA OF NORMAL INDIVIDUALS AND IN PATIENTS WITH LEUKEMIA
(mg /100 ml plasma)

| Amino acid | Normal | Before treatment | Remission |
|---|---|---|---|
| Phenylalanine | 0 71 (20) | 1 9 (35) | 1 93 (18) |
| Tyrosine* | 0 93 (17) | 1 50 (35) | 1 89 (15) |
| Isoleucine | 0 71 (11) | 1 15 (39) | 1 07 (18) |

Significant differences Nonsignificant differences methionine histidine arginine tryptophan leucine glutamic acid threonine lysine and valine Figures in parentheses indicate number of cases studied

were found in the leukemic patients when compared to normal for three of the amino acids—phenylalanine tyrosine and isoleucine This was an intriguing finding and we were stimulated to corroborate these data and to explain the role of these amino acids in the leukemic process

Recently it has been possible to extend these data and as a further check on the microbiological assays a chemical method for detecting tyrosine in blood was performed according to the method of Udenfriend and Cooper (23) With 1 nitroso 2 naphthol compounds having a terminal phenyl hydroxyl group reacted to give so called tyrosyl" values The tyrosyl" values were determined in both normal children and normal adults as well as in leukemic patients in these age groups As will be seen from Table II the children with acute leukemia have higher

no significant differences exist in the adult type of leukemia and in the cancer patient but it is of distinct interest that the arginine values in children with acute leukemia appear to be higher than those in normal children These findings are in contrast to that obtained by microbio logical assay The import of elevated arginine in plasma of children is *not clear Additional information is now being sought on this amino* acid

## Excretion of Urinary Amino Acids

A large number of 24 hour urine samples obtained from leukemic patients were analyzed for both creatine and creatinine This study was undertaken to find out whether the increased metabolic rate described earlier was reflected in the breakdown of protein or in abnormal liver function If the catabolic processes were sufficiently accelerated one might suspect that the creatinine and creatine values would be appreci ably higher than in normal individuals Since it has already been shown *that in children at least* the arginine blood levels were elevated it might be speculated that this creatinine precursor could have been the basis for the increased values An unknown factor in this particular experiment however was the degree of liver involvement by the leu kemic process and the ability of this organ to metabolize creatinine may have influenced the results

In a previous paper (27) the difficulties involved in these determina tions were described and the variable results were commented on It had been shown by Dinning and Seager (8) that the creatininuria which was observed in leukemic mice could be directly correlated with the in crease in the white blood cells

In view of the elevated plasma tyrosine levels it was *of interest to* determine the excretion of urinary tyrosine Twenty four hour urine samples were collected on a group of patients with leukemia prior to therapy and after the administration of folic acid antagonists Urine was preserved with toluol and brought to the laboratory after the com pletion of the 24 hour sample From experience we had found that urinary collection on a pediatric metabolic ward can best be done by having periods divided according to the nurses working day and ac cordingly three equal 8 hour periods were pooled in order to facilitate accuracy of sample collection The analytical method of Medes (15) was used and the tyrosyl values represent many of the metabolic prod ucts of tyrosine As was shown in a previous publication (27) the ele vated plasma tyrosine values are not reflected in any increase in urinary tyrosyl No further information is available and it is obvious that

the dermis The presence of DOPA oxidase could also be demonstrated in skin by appropriate staining techniques (6) This finding could supposedly be related to the increased amount of circulating phenylalanine but this appears to be a dubious explanation for increased melanogenesis However support for the apparent relationship between both tyrosine and phenylalanine and folic acid metabolism has been demonstrated by a number of workers (10 16 31) There is no question but that folic acid metabolism is abnormal in leukemia and is concerned with nucleic acid formation The skin hyperpigmentation in leukemic patients treated with a folic acid antagonist is not completely explained despite much work done on the enzymatic oxidation of tyrosine and its oxidation products in the formation of melanin

At the present time although hyperpigmentation and elevated phenylalanine and tyrosine levels occur in the same patient no direct proof is available that melanogenesis increases because of alteration in the tyrosine metabolic pathway The relationship is very suggestive however and further work on this point seems necessary

In Tables IV and V the arginine values in children and adults are listed The method used was that of Gilboe and Williams (personal communication) It will be seen from an examination of the data that

TABLE IV
ARGININE IN PLASMA IN NORMAL AND LEUKEMIC CHILDREN

| Classification | Number of patients | Arginine μg/ml plasma | S D | P |
|---|---|---|---|---|
| Normal | 7 | 12 6 | 4 2 | —— |
| Leukemic | 9 | 16 0 | 3 4 | 0 05 |

TABLE V
ARGININE IN PLASMA OF NORMAL, LEUKEMIC AND CANCEROUS ADULTS

| Classification | Number of patients | Arginine μg/ml plasma | S D | P |
|---|---|---|---|---|
| Normal | 7 | 15 0 | 2 8 | —— |
| Chronic granulocytic leukemic | 19 | 15 7 | 3 4 | > 0 5 |
| Chronic lymphatic leukemic | 10 | 18 5 | 2 7 | > 0 5 |
| Subacute leukemic | 3 | 18 3 | — | > 0 5 |
| Cancerous | 26 | 19 4 | 4 2 | > 0 5 |
| Noncancerous nonleukemic | 10 | 17 6 | 6 0 | > 0 5 |

no significant differences exist in the adult type of leukemia and in the cancer patient but *it is of distinct interest that the arginine values in* children with acute leukemia appear to be higher than those in normal children These findings are in contrast to that obtained by microbiological assay The import of elevated arginine in plasma of children is not clear Additional information is now being sought on this amino acid

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additional data are needed to define the metabolic activity of this amino acid

A recent preliminary study by Lo Bianco and Sala (13) reported on amino aciduria in nine leukemic patients Using a combination of the chromatographic method for urinary amino acids a method for α NH nitrogen in the blood and the Kjeldahl method for total N in the urine they found that the α amino N of the blood from some leukemic children was indeed higher and that the urinary excretion of some amino acids was increased when compared to values from normal children of the same age As far as could be determined from Lo Bianco and Sala s work it is likely that those amino acids that are elevated in the blood also appear in the urine Certainly tubular reabsorption phenomena and liver function play a part in these findings These workers found that therapy had no influence on either the blood or urinary findings No differences were observed in the leukemic patients whether they were in the acute or in the remission phase of the disease This confirms our microbiological data which showed (27) that remission of the disease *did not alter the level of free amino acids in the plasma*

## Glutamic Acid Dehydrogenase

The knowledge concerning metabolic changes that take place in white blood cells from normal and leukemic subjects has been summarized in several reviews (4 9 24 25) but only meager information is available on those enzymes concerned with amino acid metabolism As part of a comprehensive study on these enzymes initial efforts were directed to the determination of glutamic acid dehydrogenase and glutamic oxal acetic acid transaminase two of the enzymes concerned with glutamic acid metabolism

These enzymes seemed appropriate to study since it was shown by Beaton *et al* (3) and by White *et al* (29) that plasma levels of free glutamic acid were increased in human subjects with malignant growths and that chick embryos with carcinoma implanted into their yolk sacs showed an increased content of glutamic acid (30) White blood cells were separated from heparinized blood after rouleaux formation had been induced in red cells by the use of 0 8% dextran The majority of the white cells were recovered in the supernatant plasma Despite the best separations some red cells contaminated the white cell preparation but fortunately red blood cells do not contain either enzyme and so the enzyme values reflect only white blood cell activity

Glutamic acid dehydrogenase was determined by a manometric technique to suit the conditions under study (26) Table VI lists in brief

*form the glutamic acid dehydrogenase activity in white blood cells* Despite the variation in white count found in these groups of patients the dehydrogenase value for any individual patient was constant The mean activity expressed as change in optical density $\times 10^3/2 \times 10^7$ white blood cells in 30 minutes was four times as high as in normal controls or in a group on noncancer hospital cases It should be stressed that the enzyme activity within each group of patients varied considerably and no adequate explanation for this variation can now be offered *It was of distinct interest that the glutamic acid dehydrogenase value in* white blood cells was not affected by therapy Typical values before and after treatment are presented in Table VII

TABLE VI
GLUTAMIC ACID DEHYDROGENASE ACTIVITY IN WHITE BLOOD CELLS

| Classification | Number of cases | WBC count, $\times 10^3$ | Mean activity | Range | S D |
|---|---|---|---|---|---|
| Acute leukemic | 9 | 14–15 | 63† | 37–137 | 34 |
| Chronic lymphatic leukemic | 10 | 3 2–377 | 62† | 0–188 | 54 |
| Chronic granulocytic leukemic | 14 | 6 9–500 | 61† | 0–183 | 53 |
| Cancerous | 28 | | 64† | 0–180 | 39 |
| Nonleukemic hospital patients | 16 | | 14 6 | 0– 61 | 17 |
| Normal | 14 | | 17.0 | 0– 50 | 15 |

Change in optical density $\times 10^3/2 \times 10^7/30$ min
† Highly significant $P = < 0\ 01$

TABLE VII
EFFECT OF THERAPY ON WHITE BLOOD CELL GLUTAMIC ACID DEHYDROGENASE ACTIVITY IN LEUKEMIA

| Diagnosis | Treatment | Days of treatment | ΔO D$_{30}$ Before | After |
|---|---|---|---|---|
| Acute | Frequent transfusions | 7 | 50 ( 18) | 7 ( 10) |
| Chronic granulocytic | X ray | 4 | 64 (218) | 44 (154) |
| Chronic granulocytic | Myleran† | 4 | 40 ( 5) | 36 ( 28) |
| Chronic granulocytic | X ray | 13 | 93 (241) | 94 (410) |
| Chronic granulocytic | OPSPA‡ and transfusions | 23 | 12 ( 59) | 0 ( 73) |

Values in parentheses are WBC counts
† 1 4 Dimethanesulfonyloxybutane
‡ Oxapentamethylenediethyleneth ophosphoram de

additional data are needed to define the metabolic activity of this amino acid

A recent preliminary study by Lo Bianco and Sala (13) reported on amino aciduria in nine leukemic patients Using a combination of the chromatographic method for urinary amino acids a method for α NH nitrogen in the blood and the Kjeldahl method for total N in the urine they found that the α amino N of the blood from some leukemic children was indeed higher and that the urinary excretion of some amino acids was increased when compared to values from normal children of the same age As far as could be determined from Lo Bianco and Sala s work it is likely that those amino acids that are elevated in the blood also appear in the urine Certainly tubular reabsorption phenomena and liver function play a part in these findings These workers found that therapy had no influence on either the blood or urinary findings No differences were observed in the leukemic patients whether they were in the acute or in the remission phase of the disease This confirms our microbiological data which showed (27) that remission of the disease did not alter the level of free amino acids in the plasma

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These enzymes seemed appropriate to study since it was shown by Beaton *et al* (3) and by White *et al* (29) that plasma levels of free glutamic acid were increased in human subjects with malignant growths and that chick embryos with carcinoma implanted into their yolk sacs *showed an increased content of glutamic acid* (30) White blood cells were separated from heparinized blood after rouleaux formation had been induced in red cells by the use of 0 8% dextran The majority of the white cells were recovered in the supernatant plasma Despite the best separations some red cells contaminated the white cell preparation but fortunately red blood cells do not contain either enzyme and so the enzyme values reflect only white blood cell activity

Glutamic acid dehydrogenase was determined by a manometric tech nique to suit the conditions under study (26) Table VI lists in brief

more complete data fail to substantiate this claim Chemotherapy or x irradiation had no effect on the white blood cells transaminase values (Table X) or on plasma transaminase values (Table XI)

TABLE VIII
GLUTAMIC OXALACETIC ACID TRANSAMINASE ACTIVITY OF WHITE BLOOD CELLS

| Classification | Number of cases | Range of WBC × $10^3$ | Mean transaminase activity µl CO | S D | P |
|---|---|---|---|---|---|
| Acute leukemic | 10 | 14–150 | 58 | 27 | > 0.9 |
| Chronic lymphatic leukemic | 7 | 59–377 | 84 | 30 | > 0.9 |
| Chronic granulocytic leukemic | 12 | 18–241 | 63 | 25 | > 0.9 |
| Nonleukemic noncancerous | 17 | 7– 17 | 50 | 41 | > 0.9 |
| Normal | 5 | 5– 7 | 72 | 26 | > 0.9 |

Microliters CO equivalent to oxalacetate transaminated per 2 × $10^7$ WBC per hour

TABLE IX
GLUTAMIC OXALACETIC ACID TRANSAMINASE IN PLASMA OF PATIENTS WITH LEUKEMIA AND CANCER

| Classification | Number of patients | Mean transaminase activity µl $CO_2$ | S D |
|---|---|---|---|
| Leukemic | 34 | 22 | 27 |
| Cancerous | 29 | 13 | 11 |
| Nonleukemic noncancerous | 24 | 12 | 16 |
| Normal | 7 | 12 | 16 |

Microliters CO equivalent to oxalacetate transaminated per 0.2 ml of plasma per hour

*Note* P value for comparison of leukemic or cancerous with nonleukemic non cancerous was > 0.05

### Role of Ethionine in Transplantable Mouse Leukemia

It seems conceivable that if an essential amino acid is not available for synthesis of a protein to be used as a specific enzyme the particular enzyme may not be formed If this enzyme is necessary for the elaboration of nucleic acids it may be postulated that in the presence of an amino acid antagonist these vital nuclear substances will not be formed Whether this circumstance occurs in the intact animal is only speculation

Figure 1 presents a detailed study of white blood cell glutamic acid dehydrogenase and the white cell count in a two year old child with chronic granulocytic leukemia given a variety of antileukemic drugs The enzyme activity was unaffected by a variety of agents but after OPSPA therapy[1] the enzyme value fell A month later just prior to death the enzyme value rose to a maximum Examination of these data also demonstrate that the peripheral white count had little effect on this enzyme since the enzyme activity was high when the white cell count was low Just before death the enzyme value was low despite the high white blood cell count

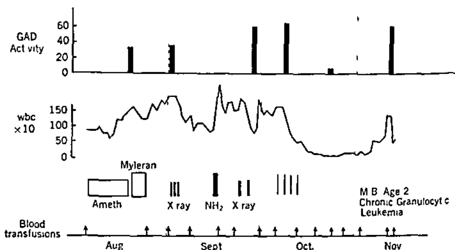



FIG 1 Effect of therapy on glutamic acid dehydrogenase

## Glutamic Oxalacetic Transaminase

This enzyme was determined manometrically by the procedure of Ames and Elvehjem (1) with pyridoxal phosphate added as recommended by O'Kane and Gunsalus (19)

The glutamic oxalacetic transaminase values in white blood cells (Table VIII) and in plasma (Table IX) are presented In 29 cases of the several types of leukemia the mean transaminase value was about equal to that in normal or in nonleukemic hospital patients Although early data suggested that the transaminase in white blood cells from patients with chronic lymphatic leukemia was higher than in controls

1 The OPSPA was kindly provided by Dr C Heidelberger

labeled methionine or glycine into rat proteins and they suggested that ethionine could prevent conversion of methionine to cystine At about the same time Stekol *et al* (22) used ethyl labeled ethionine and dem onstrated that this ethyl group was found in the trimethylamino portion of choline This indicated that the ethyl group of ethionine was replac ing methyl groups in the transmethylating process With this evidence that ethionine probably interferes with methionine utilization we used ethionine with several strains of mouse leukemia The antagonist was either fed or injected and in all experiments the leukemic spleen cells were transplanted by the intraperitoneal route Since the antagonists cause distinct weight loss it was necessary to resolve the question whether manition might play a role in inhibiting the transplants

Experiments showed that simple caloric restriction per se did not interfere with the incidence of leukemia Series 18 and 19 shown in Table XII demonstrate that limiting² the food intake to 1 25 g per day

TABLE XII
FB Mice (Series 18 and 19)

| Group | Number of leukemic deaths | |
|---|---|---|
| | Adult | Young |
| Rockland diet + 9-6 leukemia | 7/9 (77%) | 6 10 (60%) |
| Rockland (restrict d to 1.25 g/day) + 9-6 leukemia | 11/19 (73%) | 9/13 (69%) |
| Rockland + 0 75% DL-ethionine + 9-6 leukemia | 7/12 (58%) | 4/9 (44%) |

(one fourth to one third the normal intake) did not reduce the incidence of the leukemia in two separate experiments using both young and old FB mice The younger mice showed a greater reduction in leukemia than did the older mice of the FB strain It having thus been shown that caloric restriction did not interfere with the incidence of leukemia it was possible to set up experiments that could test the influence of ethionine on mouse leukemia The addition of ethionine to the diet was able to decrease the incidence of death from leukemia in this strain as shown in Table XII

Further experiments were able to test a number of factors that were related to the time of ethionine administration Table XIII shows that giving the ethionine for 3 days prior to the leukemic transplant made no difference in the "take" of the transplant When the leukemia was trans

² These mice were obtained from random breeding of FB male mice with any $F_1$ hybrid female

Experiments were devised to test whether the amino acid antagonists could be effective in delaying the onset of or the inhibition of transplantable leukemia in selected strains of mice Although the number of

TABLE X

EFFECT OF THERAPY ON WHITE BLOOD CELL GLUTAMIC OXALACETIC TRANSAMINASE ACTIVITY IN LEUKEMIA

| Diagnosis | Treatment | Interval | Mean transaminase activity µl $CO_2$* After | Before |
|---|---|---|---|---|
| Acute | 6 MP† | 4 months | 84 | 85 |
| Acute | Amethopterin | 32 days | 92 | 104 |
| Chronic granulocytic | Transfusions | 3 months | 38 | 45 |
| Chronic granulocytic | X ray | 18 days | 42 | 49 |
| Chronic granulocytic | OPSPA‡ | 25 days | 26 | 0 |

* Microliters $CO_2$ equivalent to oxalacetate transaminated per $2 \times 10^7$ WBC per hour

† 6 Mercaptopurine

‡ Oxapentamethylenediethylene thiophosphoramide

TABLE XI

EFFECT OF THERAPY ON PLASMA GLUTAMIC OXALACETIC TRANSAMINASE ACTIVITY IN LEUKEMIA

| Diagnosis | Treatment | Days of treatment | Mean transaminase activity µl $CO_2$* Before | After |
|---|---|---|---|---|
| Chronic lymphocytic | TEM† | 56 | 60 | 40 |
| Chronic granulocytic | Myleran‡ | 5 | 61 | 52 |
| Chronic granulocytic | X ray | 27 | 0 | 3 |

* Microliters $CO_2$ equivalent to oxalacetate transaminated per 0 2 ml of plasma per hour

† Triethylene melamine

‡ 1 4 Dimethanesulfonyloxybutane

amino acid antagonists were limited we first used DL ethionine because more was known about its metabolic activity At about the time that we began to study the metabolic and nutritional effects of ethionine Simpson *et al* (21) showed that this antagonist inhibits incorporation of

group than in the ethionine groups The data in Table XV show the delayed incidence of death in the mice receiving ethionine

These data imply that ethionine actually interferes with the formation of protein essential to the leukemic cell This is accomplished probably by incorporation of the ethionine into the protein molecule as shown by Stekol *et al* (22) or by the effect of ethionine on protein synthesis by inhibiting the incorporation of methionine and glycine into

TABLE XV
FB STRONG A (SERIES 35)

| | Day of mouse death after transplant | | | | | | |
|---|---|---|---|---|---|---|---|
| | 9 | 11 | 13 | 14 | 15 | 16 | 17 |
| Rockland + 926 leukemia | 2/10 | | 3/10 | 7/10 | | | |
| Rockland + 20 mg DL-ethionine + 926 leukemia | 1/9 | | | | 3/9 | 5/9 | 8/9 |

liver protein (21) The question remains Is the methionine important structurally for synthesis of a vital enzyme necessary for cell proliferation or does the antagonist actually prevent the dysplastic process at a stage where incorporation of essential nuclear protein is vital? At the present time these speculations must serve as the basis for future experimentation

## Summary

The data presented here support the conclusion that amino acids undoubtedly play a part in the pathophysiology of the leukemias At the moment the elevated tyrosine level in the blood of leukemic patients holds the greatest interest. From our study of urinary amino acid excretion we feel that the elevated tyrosine level that occurs in plasma may not be reflected in the tyrosine excreted in the urine Further work is needed to elucidate the role of this amino acid in the dysplastic process

Enzymes concerned with amino acid anabolism or catabolism in the white blood cells appear to have significance since there is apparently no question that glutamic acid dehydrogenase metabolism is abnormal in the white cells of patients with leukemia Additional work on other white cell enzymes is now in progress The use of amino acid antagonists in either human or mouse leukemia seems a profitable way to study some of the aberrations that occur in this disease process In transplantable mouse leukemia we have been able to show that by means of its dietary effect ethionine can inhibit the onset of leukemia in certain strains of mice Adequate controls demonstrate that caloric restriction per se does not interfere with the incidence of leukemia

planted 3 days prior to the first ethionine administration again no significant differences were observed The administration of ethionine intraperitoneally every other day was also not impressive The greatest reduction in the number of mice showing positive leukemia was found in groups 4 and 8 both of which received optimum ethionine either concurrently with or for 4 to 6 weeks prior to the leukemic transplant It

TABLE XIII

NUMBER OF FB ADULT MICE SHOWING POSITIVE LEUKEMIA (SERIES 24)

| | | |
|---|---|---|
| 1 | Rockland + 926 leukemia | 12/15 |
| 2 | Restricted Rockland + 926 leukemia | 10/15 |
| 3 | Rockland + 20 mg ethionine i p + 926 leukemia 72 hours later | 9/9 |
| 4 | Rockland + 20 mg ethionine + leukemia | 11/19 |
| 5 | Rockland + 0 75% DL ethionine + 926 leukemia | 11/15 |
| 6 | Rockland + 926 leukemia + 20 mg DL-ethionine i p started 72 hours later | 11/15 |
| 7 | Rockland + 20 mg ethionine every other day | 11/15 |
| 8 | Rockland + 20 mg ethionine i p for 4 to 6 weeks prior to 926 leukemia | 11/20 |

is difficult to define the role of the antagonist but from many experiments using FB randomly bred mice it is possible to conclude that ethionine appears to decrease the incidence of leukemia to some degree in several strains of mice With $F_1$ hybrids of the FB strain or with AK or FB Strong A mice the results appear to be more definitive since ethionine represses the leukemia more consistently In Table XIV

TABLE XIV

NUMBER OF MICE WITH POSITIVE LEUKEMIA (SERIES 34)

| | |
|---|---|
| Rockland plus AK leukemia | 17/17 |
| Rockland plus 12 mg DL ethionine plus AK leukemia | 4/11 |

animals from the AK strain had less leukemia in ethionine injected groups than the controls The results in this series were again checked by gross and by microscopic examination and the leukemia identified morphologically

Another group of data on the delaying action of ethionine was demonstrated in FB Strong A hybrids Although the data at the end of the experiment showed nearly identical incidence of leukemia the enlarged spleens were easily palpated at least 6 days earlier in the non ethionine

# 20

# Urinary Amino Acid Excretion in Leukemia

JORGE AWAPARA
The University of Texas M D Anderson Hospital and
Tumor Institute Houston Texas

## Introduction and Methods

Urine contains in addition to the well known substances a large number of substances present in smaller quantities It is not unusual to find a product or products of catabolism that are present only in the urine of patients with a certain disease This work was started in the hope that some clues could be found in the urine of leukemic patients if an exhaustive analysis could be carried out For this purpose paper chromatography is an ideal method because one can detect simultaneously a large number of substances The method permits the investigator to explore new areas For example it is not uncommon to find a new compound in some biological fluid or tissue This leads almost invariably to the discovery of new reactions new enzymes and new concepts in metabolic pathways When the method of paper chromatography was adapted for the study of urine technical difficulties were encountered at once As a point of departure in this study we decided to measure the free amino acids and other related substances (taurine glutamine glutathione etc) which are detected by the same procedure In order to do this it was necessary to remove from the urine substances like urea and inorganic salts which interfere with the method Moreover to detect as many amino compounds as possible the urine was concentrated fivefold prior to chomatographic analysis The details for the preparation of urine specimens prior to chromatography have been published (1) Briefly the method consists in removing pigments and large molecules by treatment of the urine with activated charcoal in the presence of some chloroform The charcoal treated urine is then desalted by means of ion exchange resins A solvent system suitable

Acknowledgments

This work was largely aided by Sterling A Copeland Carl Monder and J J Kelley each of whom contributed a portion of the laboratory data Financial assistance was provided in part by grants from the National Institutes of Health the Playtex Park Research Institute the American Cancer Society through the Committee on Growth of the National Research Council the Fairchild Foundation and the Wisconsin Alumni Research Foundation

REFERENCES

1 Ames S R and Elvehjem C A *J Biol Chem* **166** 81 (1946)
2 Baldridge C W and Barer A *Arch Internal Med* **51** 589 (1933)
3 Beaton J R McGanity W J and McHenry E W *Can Med Assoc J* **65** 219 (1951)
4 Beck W S and Valentine W N *Cancer Research* **13** 309 (1953)
5 Becher E and Herrmann E *Munch med Wochschr* **72** 2178 (1925)
6 Becker S W *J Invest Dermatol* **5** 463 (1942)
7 Cannon M D *Science* **106** 597 (1947)
8 Dinning J S and Seager L D *Science* **114** 502 (1951)
9 Doan C A *Bull N Y Acad Med* **30** 415 (1954)
10 Govan C D Jr and Gordon H H *Science* **109** 332 (1949)
11 Grafe E *Arch Klin Med* **102** 406 (1911)
12 Krantz C I and Riddle M C *Am J Med Sci* **175** 229 (1928)
13 Lo Bianco S and Sala I *Lattanta* **25** 649 (1954)
14 Masson P *in* Biology of the Melanomas Vol 6 p 15 New York Academy of Medicine 1948
15 Medes G *Biochem J* **26** 917 (1932)
16 Morris J E Harpur E R and Goldbloom A *J Clin Invest* **29** 325 (1950)
17 Murphy J B Means J H Aub J C *Arch Internal Med* **19** 890 (1917)
18 Okada S and Hayashi T *J Biol Chem* **51** 121 (1922)
19 O Kane D E and Gunsalus I C *J Biol Chem* **170** 425 (1947)
20 Riddle M C and Sturgis C C *Arch Internal Med* **39** 255 (1927)
21 Simpson M V Farber E and Tarver H *J Biol Chem* **182** 81 (1950)
22 Stekol J A Weiss K and Weiss S *J Am Chem Soc* **72** 2309 (1950)
23 Udenfriend S and Cooper J R *J Biol Chem* **196** 227 (1952)
24 Valentine W N *Ann Rev Med* **6** 77 (1955)
25 Valentine W N *Blood* **6** 845 (1951)
26 Waisman H A Monder C and Williams J N Jr *Cancer Research* **16** 344 (1956)
27 Waisman H A Pastel R A Poncher H G *Pediatrics* **10** 653 (1952)
28 Waisman H A Richmond J B and Zimmermann A A *Proc Soc Exptl Biol Med* **75** 332 (1950)
29 White J M Beaton J R and McHenry E W *J Lab Clin Med* **40** 703 (1952)
30 White J M Ross G A L and McHenry E W *Cancer Research* **14** 173 (1953)
31 Woodruff C W Cherrington M E Stockell A K and Darby W J *J Biol Chem* **178** 861 (1949)

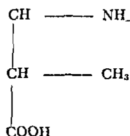
### Effect of Nitrogen Mustards on the Excretion of β Aminoisobutyric Acid

Inasmuch as little knowledge was gained by simply surveying urine specimens from leukemic patients we decided to seek new information by a different method If β aminoisobutyric acid is a product of pyrimidine catabolism we could readily infer that its presence in the urine could reflect nucleic acid breakdown To obtain more facts in support of this hypothesis we proceeded to study selected patients who were candidates for nitrogen mustard therapy Nitrogen mustard is known to cause tissue lysis and also has the advantage that is given in a single dose Patients with lymphosarcoma chronic lymphocytic leukemia and Hodgkin s disease were chosen for this study All the patients were hospitalized at the University of Texas M D Anderson Hospital and Tumor Institute and no change in the plan of treatment was made Urines were collected (24 hour collection) for several days before nitrogen mustard was given and for several days after the administration of the drug The period of study varied depending on the condition of the patient Most patients were very cooperative in this study The analyses of the urines were made by the methods described In addition to the quantitative estimation of β aminoisobutyric acid other amino acids were measured taurine glycine serine and alanine Results are shown only for β aminoisobutyric acid It is clear from the charts that nitrogen mustard (HN2) has a profound effect on the excretion of β aminoisobutyric acid The effect is of short duration and the intensity varies from patient to patient. We tried to correlate the amount of β amino isobutyric acid excreted with total leukocyte count, but there was no discernible correlation Patient E M (Fig 1) a female 55 years of age who had lymphosarcoma, received 50 mg of HN2 This patient was excreting around 500 micromoles of β aminoisobutyric acid per 24 hours prior to the administration of the mustard and her total leukocyte count was 5600 After mustard treatment her count was 3000 Notice however that after the administration of the drug her β aminoisobutyric acid excretion rose to 2257 micromoles That the rise is out of proportion to the drop in total leukocytes can be better appreciated by comparing this patient with one who had a high total leukocyte count The patient M C (Fig 2) a female 58 years old had chronic lymphocytic leukemia Her total leukocyte count prior to treatment was 33 600 Her urine was analyzed for 4 days prior to treatment and we could not find any β aminoisobutyric acid After the administration of 30 mg of HN2 the patient s count dropped to 18 000 3 days after the administration of HN2 she excreted a maximum amount of β aminoisobutyric acid This maximum was only 780 micromoles

for urinary amino acids analysis is butanol-formic acid-water (70 15 15) and lutidine-water (60 40) The method is not so accurate as one would desire since there are losses Fortunately the losses are uniform and the ratio of one component to the other remains about the same

## Excretion of Free Amino Acids in Leukemia

A series of urine specimens from children with leukemia were examined at first by the procedure outlined above The patients were at Sloan Kettering Institute Dr J Burchenal was so kind as to provide us with all the urine specimens and information for this study The results which have been reported elsewhere were disappointing except for one finding The pattern of amino acid excretion did not bear any relation to the type of disease or to the form of treatment The only rewarding information was that all the patients excreted β aminoiso butyric acid in varying amounts This in itself was interesting since β aminoisobutyric acid is not normally present in detectable amounts



β Aminoisobutyric acid

Crumpler and associates (2) were the first to report the existence of β aminoisobutyric acid in urine They examined the urine of 459 subjects of both sexes ranging in age from 3 to 50 years They found that 22 of the subjects (4 8%) excreted β aminoisobutyric acid in detectable amounts They gave no quantitative values At about the same time Fink *et al* (3) reported a similar finding They studied 140 normal subjects They found that 88 of the group excreted no detectable amounts 41 excreted an average of 300 micromoles per liter 8 excreted 400 micromoles per liter 1 excreted 2500 micromoles per liter and 2 excreted 3000 micromoles per liter It is clear from the results of Crumpler and Fink that the excretion of β aminoisobutyric acid by normal subjects is not a common event All the specimens which we have examined from leukemic patients had detectable quantities of β aminoisobutyric acid Subsequent work by Fink and associates (4 7) has demonstrated that β aminoisobutyric acid can arise from pyrimidines

mustard in the same way as patients with lymphosarcoma (Figs 7 and 8) It is true that the nitrogen mustard brought about an increase in the excretion of $\beta$ aminoisobutyric acid but the rise was not so impressive as in the patients with lymphosarcoma

It is difficult to assess the significance of this observation at the moment Many more facts are necessary to begin to understand the

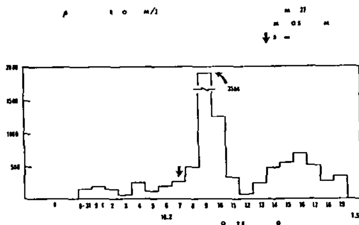


Fig 3

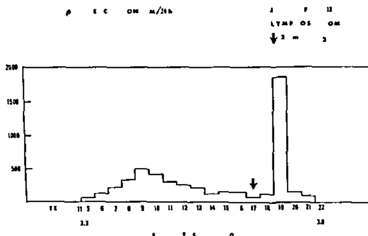


Fig 4

which is considerably less than the 2257 micromoles excreted by our first patient There seems to be little correlation between total leukocyte count and the excretion of $\beta$ aminoisobutyric acid It is not improbable that the amount of $\beta$ aminoisobutyric acid excreted depends on the amount of lymphatic tissue affected by the drug Two patients with Hodgkin s disease in this study did not respond to the nitrogen

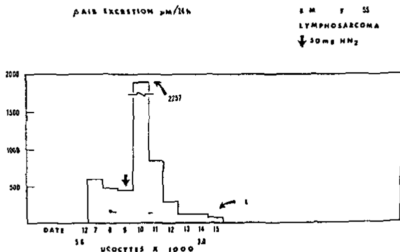


Fig 1

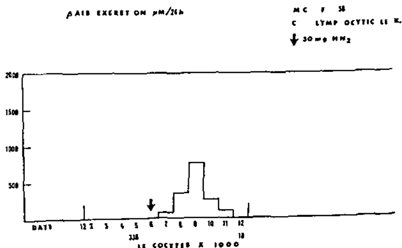


Fig 2

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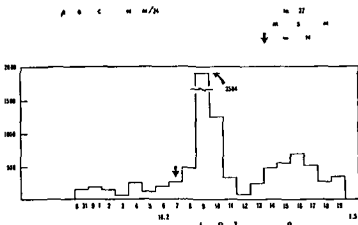


Fig 3

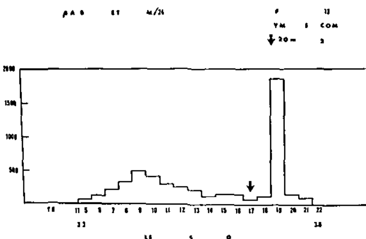


Fig 4

reasons for this phenomenon. More work is under way and we hope that when a *sufficient number of patients has been studied* an interpretation will be possible. It is indeed fortunate that while this work was underway Fink and associates (37) reported their findings on the origin of $\beta$-aminoisobutyric acid. Their results point to the fact that $\beta$-aminoisobutyric acid is formed from a thymine derivative.

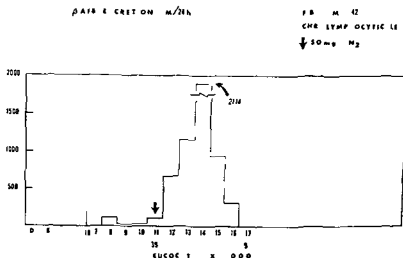



Fig 5

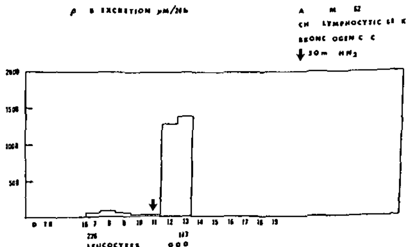



Fig 6

namely dihydrothymine The existence of dihydrothymine has not been demonstrated but in their experiments they were able to show the conversion of this compound to β aminoisobutyric acid If β aminoisobutyric acid is the end product of thymine catabolism one could explain the rise observed on the basis of increased DNA catabolism It is known that uric acid also increases in leukemia Moreover we have determined uric acid excretion in a few of our patients and found a similar pattern in the main it appears that uric acid excretion increases at the same time Since uric acid is formed from

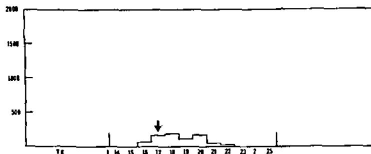
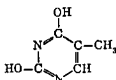


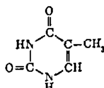

Fig 7



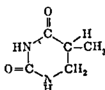

Fig 8

purines and is not catabolized further one finds it in the urine Thymine could conceivably be oxidized or perhaps converted to β amino isobutyric acid We do not know to what extent β aminoisobutyric acid is oxidized in the body If it is oxidized we are measuring in the

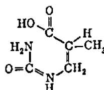


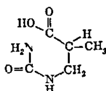

Thymine

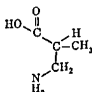



Dihydrothymine




Ureido β AIB




β AIB

urine only a small fraction of the total amount produced Work is under way to determine the extent of oxidation of β aminoisobutyric acid

## REFERENCES


1 Awapara J and Sato Y *Clin Chim Acta* **1** 75 (1956)
2 Crumpler H R Dent C E Harris H and Westall R G *Nature* **167** 307 (1951)
3 Fink K Henderson R B and Fink R M *Proc Soc Exptl Biol Med* **78** 135 (1951)
4 Fink K Henderson R B and Fink R M *J Biol Chem* **197** 441 (1952)
5 Fink R M Fink K and Henderson R B *J Biol Chem* **201** 349 (1953)
6 Fink R M McGoughey C Cline R E and Fink K *J Biol Chem* **218** 1 (1956)
7 Fink K *J Biol Chem* **218** 9 (1956)

# 21

# Free or Easily Extractable Amino Acids in Blood Cells and Body Fluids

GEORGE ROUSER[1]

Department of Biochemistry Medical Research Institute
City of Hope Medical Center Duarte California

*This is a progress report of studies dealing with the metabolism of the* formed elements of blood with particular emphasis on the leukocytes The broad aim of the project is to outline the metabolic systems of importance in blood cells in various species and the effects on these systems in certain pathological states This eventually would include the determination of the distribution of various enzyme systems and the concentrations and turnover rates of their substrates and the products of their reactions The first step in elucidating metabolic balances in living cells involves the determination of the types and amounts of various important cellular constituents The technique of paper chromatography has been selected for this purpose since it has been found to be an excellent and simple method for giving simultaneous information about a large number of variables Nitrogen metabolism has been the first area to be considered not only because of the great importance of amino acids and related products to cellular processes but because suitable methods were at hand for such studies

It has been shown that the free amino acid content of the plasma is usually much lower than that of the cells (2 10 12) and that all cells with the possible exception of erythrocytes (12 17) can concentrate many amino acids As with other metabolites the uptake of amino acids by cells may be accompanied by a loss of some substances from the cells e g Ehrlich ascites tumor cells lose potassium ions during the uptake of $\alpha,\gamma$ diaminobutyric acid (1) The complex relationship of

[1] Dr Carson H Tishkoff collaborated in studies on animal blood cells and Drs Arthur Samuels and Bohdan Jelinek collaborated in some of the studies of patients with leukemia

the concentrations of amino acids in blood cells to those in plasma which is in turn influenced by changes in the metabolic status of the organism as a whole makes it highly desirable to study both cells and plasma From an extensive survey of these relationships in this laboratory it has been concluded that a study of body fluids or cells alone may either fail to yield useful information or may actually give rise to misleading interpretations

The exact form within cells of the so called free or easily extractable amino acids is not known although if binding occurs it is of a weak type as indicated by the ease of extraction of these compounds by gentle procedures Ionic attraction van der Waals forces and hydrogen bonding may be involved in binding amino acids to substances of high molecular weight such as proteins or nucleic acids

The present report describes results of an attempt to correlate the free amino acid pattern with cell morphology in animals and man Observations also will be reported of an extensive paper chromatographic survey of blood cells and body fluids in patients with various types of leukemia which has focused attention on some specific aspects of amino acid metabolism

## Methods

Blood and marrow samples were drawn into siliconed heparinized syringes from adult animals and human beings and separation of cells was accomplished by centrifugation Clean cuts of platelets white cells and red cells could be obtained by centrifugation at an average radial distance of 7¼ inches by starting at 400 r p m and gradually increasing the speed over a period of 3 to 5 minutes to 1500 r p m which speed was continued for 3 to 7 minutes Platelets were removed with the overlying plasma and sedimented at 2500 r p m for 5 to 10 minutes White cells were usually removed as nearly quantitatively as possible along with a considerable quantity of red cells transferred to a smaller bore tube and recentrifuged This process was repeated until the contamination with red cells was reduced to a level which would not affect the chromatographic results Different types of leukocytes present in a mixture were frequently separated by the layering technique reviewed by Rouser (23) The method involves recentrifugation of the white cell mixture in a narrow bore tube aspiration of various layers and microscopic examination after supravital staining The layers found to have the desired cell type with the smallest content of other types are then examined chromatographically

All samples were first extracted with 80% ethanol After evaporation

of the alcohol the dried residues were suspended in a measured quantity of water and dialyzed to equilibrium against water and the dialyzate electrolytically desalted in the case of plasma red cells and urine in order to avoid serious interference with the chromatographic procedures Samples applied to paper usually were dried with warm air from a hair drier a procedure which was found to oxidize cysteine cystine and cysteinylglycine to the corresponding sulfonic acids but leaving glutathione "unoxidized This was preferred to peroxide oxidation in samples which were desalted because movement of oxidized glutathione was distorted by sulfates introduced during the desalting procedure Two dimensional paper chromatography in water saturated phenol and lutidine was performed in the usual manner A tracing showing the location of the commonly observed ninhydrin reactive constituents is shown in Fig 1 Photography of chromatograms was standardized so that the size reduction was the same in each case

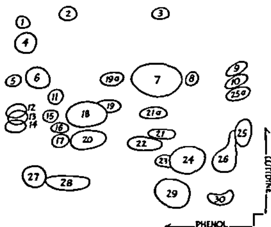



Fig 1 Map of ninhydrin reactive constituents (1) phenylalanine (2) tyrosine (3) probably tyrosine O sulfate (urine) (4) leucine + isoleucine (5) ethanolamine (6) valine (7) taurine (8) unknown (γ glutamylpeptide?) (9) cysteinylglycine (sulfonic acid) (10) cysteic acid (11) α amino-n butyric acid (12) 3-methylhistidine (urine) (13) proline (14) 1 methylhistidine (15) histidine (16) unknown (present in erythrocytes) (17) glycerylphosphorylethanolamine (18) alanine (19) threonine (common position) (19a) threonine position with some batches of Whatman No 1 paper (20) glutamine (21) serine (common position) (21a) serine position with some batches of Whatman No 1 paper (22) glycine (23) unknown (γ glutamylpeptide?) (24) glutamic acid (25) "oxidized glutathione (H O oxidized) (25a) also oxidized glutathione (26) aspartic acid (27) arginine (28) lysine (29) ethanolamine O phosphate and (30) glutathione (no H O )

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from patients carefully selected for the presence of the particular cell populations in the peripheral blood Comparisons for human subjects were made only in those instances in which the plasma and urine free amino acid patterns did not show gross differences As in the case of normal animals and man the pattern of each cell type was found to be characteristically different Figures 10 and 11 show the free amino acid patterns obtained from preparations of small and large lymphoblasts respectively of a dog with spontaneous leukemia The two types of lymphoblasts were separated by the layering technique The chromatograms are nearly identical It would therefore appear that size variation in the cells is not correlated with any notable differences in amino acid pattern Chromatograms of extracts of lymphoblasts from dog (Figs 10 and 11) and from a patient with lymphoblastic leukemia (Fig 12) are readily distinguished. Most characteristic are the differences in ethanolamine O phosphate and alanine Rodent lymphoblasts can also be distinguished from those of dog and man Human myeloblasts (Fig 13) possess a pattern that differs clearly from myelocytes (preparation including promyelocytes myelocytes and metamyelocytes Fig 14) The most characteristic differences are the very low levels of glutamine glutathione and cysteine (cystine) in the myeloblasts Myelocytes (Fig 14) have a grossly different pattern from polymorphonuclear leukocytes (Fig 15) the latter cells containing very low levels of glutamine and ethanolamine O phosphate The myelocytes were the only cells in the above series containing relatively high concentrations of glutamine

It has been observed that there is greater variation in the free amino acid patterns among cells of the myeloid series than those of the lymphoid series Small, medium and large lymphocytes have very similar patterns particularly when the nuclear-cytoplasmic ratio is approximately the same There are smaller differences between lymphocytes (Figs 19 27 30 33) and lymphoblasts (Fig 12) than between myeloblasts (Fig 13) and polymorphonuclear leukocytes (Figs 15 and 16)

3 *Amino Acid Patterns in the Same Cell Type Obtained from Normal Human Subjects and from Patients with Leukemia or Malignant Disease* The question frequently arises whether the circulating leukocytes in leukemic patients have an abnormal metabolic pattern Since it has been shown that each cell type has a characteristic free amino acid pattern only morphologically similar cells can be compared Because of the complex interrelationship between plasma and intracellular free amino acids it is necessary to compare the cells at a time when the plasma levels of the various free amino acids are as nearly identical as possible Figures 16 to 18 show the best comparisons we have obtained

**Results**

*1 Characteristic Pattern of Free or Easily Extractable Amino Acids Found in Each Type of Formed Elements of the Blood of a Particular Species* Figures 2 to 5 show chromatograms prepared from extracts of suitable quantities of various types of rabbit cells The free amino acid level in lymphocytes obtained from lymph node (Fig 2) is higher than that of the pseudoeosinophilic polymorphonuclear leukocytes obtained from peritoneal exudates (Fig 4) One difference between the two types of cells in all species studied is the higher concentration of ethanolamine O phosphoric acid in lymphocytes Large mononuclear phagocytes (macrophages) obtained from peritoneal exudates (Fig 3) contain various essential amino acids such as the leucines valine threonine and lysine that are low or undetectable in lymphocytes or the cells of the myeloid series The content of histidine is relatively high also Blood platelets (Fig 5) also show a characteristic pattern of amino acids

Figures 6 to 9 show some patterns obtained from the dog and normal man A preparation composed largely of human neutrophilic polymor phonuclear leukocytes (Fig 6) can be compared with one from dog neutrophilic polymorphonuclear leukocytes (Fig 8) A marked over all similarity can be observed The apparent species variations indicated by differences in the concentrations of glutamine glutathione cysteic acid and cysteinylglycine are within the range seen from one prepara tion to another in man Another preparation (Fig 18) of human granulocytes is almost indistinguishable from that of the dog granulo cytes Human blood platelets (Fig 7) in turn have a characteristic pattern readily distinguishable from the other blood elements The free amino acid pool is much lower in the red cells (Fig 9) than in other formed elements of blood Figure 9 was prepared using an extract corresponding to ten times the packed cell volume used for leukocytes and platelets The red cell pattern has been observed to reflect rather closely the free amino acid composition of the plasma both qualitatively and quantitatively Red cell aspartic and glutamic acid content is al ways higher than plasma however and red cells contain considerable quantities of glutathione a substance not detectable in plasma by these procedures

The above results are in keeping with the findings of Roberts and Frankel (21) that in a particular animal species each tissue of the adult organism has a characteristic pattern of free or easily extractable nin hydrin reactive constituents

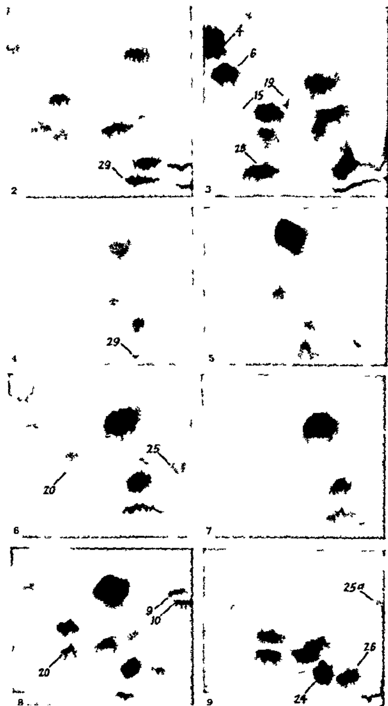
*2 Amino Acid Patterns of Leukocytes of Animals and Patients with Leukemia* Cell preparations were obtained from leukemic animals and

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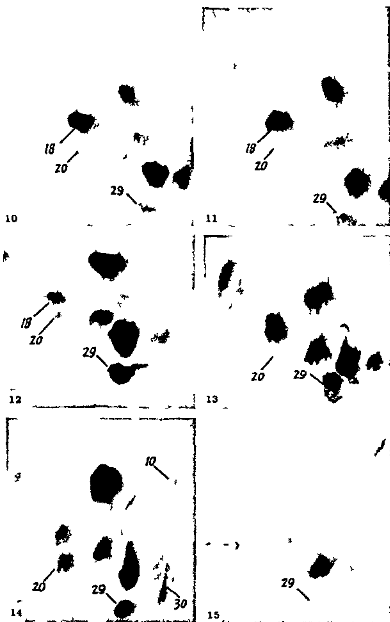
It has been observed that there is greater variation in the free amino acid patterns among cells of the myeloid series than those of the lymphoid series Small, medium and large lymphocytes have very similar patterns particularly when the nuclear-cytoplasmic ratio is approximately the same There are smaller differences between lymphocytes (Figs 19 27 30 33) and lymphoblasts (Fig 12) than between myeloblasts (Fig 13) and polymorphonuclear leukocytes (Figs 15 and 16)

3 *Amino Acid Patterns in the Same Cell Type Obtained from Normal Human Subjects and from Patients with Leukemia or Malignant Disease* The question frequently arises whether the circulating leukocytes in leukemic patients have an abnormal metabolic pattern Since it has been shown that each cell type has a characteristic free amino acid pattern only morphologically similar cells can be compared Because of the complex interrelationship between plasma and intracellular free amino acids it is necessary to compare the cells at a time when the plasma levels of the various free amino acids are as nearly identical as possible Figures 16 to 18 show the best comparisons we have obtained

FIGS 2–9 Comparison of free amino acid patterns of some cells of normal rabbit dog and man Extracts were chromatographed corresponding to the following quantities of cells (2) 35 mg rabbit lymphocytes (lymph node) (3) 70 mg rabbit large mononuclear phagocytes (monocytes from peritoneal exudates) (4) 35 mg rabbit pseudoeosinophilic polymorphonuclear leukocytes (peritoneal exudates) (5) 70 mg rabbit blood platelets (6) 40 mg human neutrophilic polymorphonuclear leukocytes (7) 40 mg human blood platelets (8) 75 mg dog neutrophilic polymorphonuclear leukocytes and (9) 1 ml packed human red blood cells


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FIGS 2–9 Comparison of free amino acid patterns of some cells of normal rabbit dog and man Extracts were chromatographed corresponding to the following quantities of cells (2) 35 mg rabbit lymphocytes (lymph node) (3) 70 mg rabbit large mononuclear phagocytes (monocytes from peritoneal exudates) (4) 35 mg rabbit pseudoeosinophilic polymorphonuclear leukocytes (peritoneal exudates) (5) 70 mg rabbit blood platelets (6) 40 mg human neutrophilic polymorphonuclear leukocytes (7) 40 mg human blood platelets (8) 75 mg dog neutrophilic polymorphonuclear leukocytes and (9) 1 ml packed human red blood cells



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FIGS 10–15 Comparison of free amino acid patterns of some cells from dog and human leukemias Extracts corresponding to the following quantities of cells were employed (10) 100 mg dog small lymphoblasts (11) 100 mg dog large lymphoblasts (12) 100 mg human lymphoblasts (13) 100 mg human myeloblasts (14) 100 mg mixed myelocytes (promyelocytes metamyelocytes and myelocytes) (15) 100 mg neutrophilic polymorphonuclear leukocytes

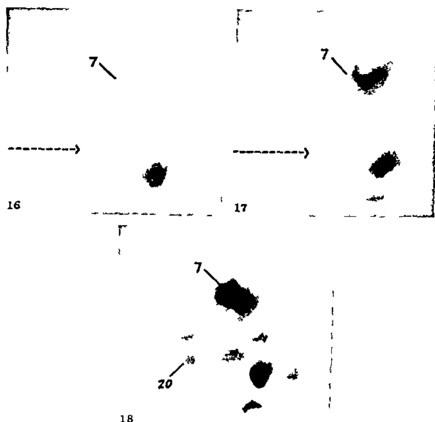
grams of the extracts shown in Figs 16 and 17 It cannot be stated at present that the differences observed are a consequence of metabolic alterations in the cells examined On the other hand the difference may be a consequence of alterations of taurine and glutamine levels in the plasma since it has been found generally that greater amounts of these substances are found in leukocytes when the concentrations in plasma and urine are higher This was actually the case in the patients whose cells were examined above

4 *Glutamine and Glutathione Metabolism in the Leukemias* Abnormally low glutamine content has been observed in the plasma of a patient with chronic granulocytic leukemia containing primarily polymorphonuclear leukocytes in the peripheral blood but not in patients with chronic granulocytic leukemia who had large numbers of immature cells in the circulation A tendency toward a lowered plasma glutamine level was observed in acute myeloblastic and lymphoblastic and chronic lymphocytic leukemias and in the body fluids of patients with carcinoma of the breast Hodgkin s disease lymphosarcoma and reticulum cell sarcoma

Free amino acid patterns illustrating the virtual absence of glutamine in a patient with chronic lymphocytic leukemia are shown for the small lymphocyte (Fig 19) red cells (Fig 20) plasma (Fig 21) and urine (Fig 22) respectively In addition to the decrease in glutamine increases in glutamic and aspartic acids and in alanine have been noted in some of the cases For comparison chromatograms of normal plasma and urine are shown in Figs 23 and 24 When untreated patients were studied at various intervals occasionally normal plasma and urine patterns were obtained at other times large deviations from the normal picture could be observed Plasma glutamine has been found to return toward normal levels in some fasted patients after treatment with TEM or nitrogen mustard

Cysteinylglycine detectable on chromatograms probably as the sulfonic acid diketopiperazine was observed frequently in small amounts in lymphocytes and in larger quantities in granulocytes and platelets Chromatograms regularly showed that the quantity of glutathione varied inversely with the content of cysteine (cystine) and cysteinylglycine Two new compounds tentatively identified as γ glutamylglutamine and γ glutamylcysteine were found occasionally in platelets The presence of these compounds was also associated with a low level of glutathione Although some of the variability noted in the content of the various sulfur compounds was probably attributable to the unavoidable delay in preparing some samples there appears to be some variation in the

to date Figure 16 was prepared from an extract obtained from a patient with granulocytic leukemia having almost exclusively neutrophilic polymorphonuclear leukocytes in the blood The chromatogram in Fig 17 was obtained from an extract of morphologically similar cells separated




FIGS 16-18 Comparison of amino acid patterns of neutrophilic polymorphonuclear leukocytes of human subjects (16) granulocytic leukemia (17) reticulum cell sarcoma and (18) normal Extracts equivalent to 100 mg of material were employed in each case

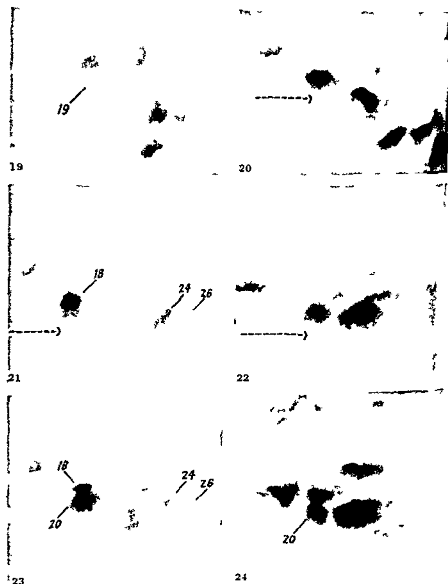
by the layering technique from the peripheral blood of a patient with reticulum cell sarcoma The pattern shown in Fig 18 is from a preparation of neutrophilic polymorphonuclear leukocytes from a normal individual The preparation from the normal individual (Fig 18) showed considerably larger quantities of taurine than the other two samples and the presence of glutamine which was not detected on the chromato

creas was also present in the formed elements of the blood and more detailed studies were carried out to check this possibility

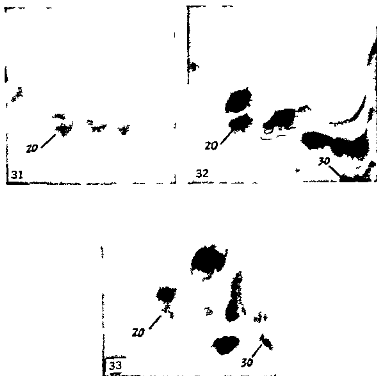
A patient with chronic lymphocytic leukemia with a cell count of 100 000/mm³ and a differential count of 95% small lymphocytes was injected with 15 mg/kg of L(+) glutamine via the femoral artery and blood samples were withdrawn from the same site at 5 15 35 60 90 and 120 minutes Figures 25 to 33 show the findings in the plasma, red cells and white cells before the injection and at 5 minutes and 60 minutes thereafter After injection the plasma glutamine level (Fig 28) was elevated above the initial level (Fig 25) to only a relatively small extent and at 120 minutes it had decreased to approximately its original level Elevation of red cell glutamine was noted during the experiment but the most marked change was the almost complete disappearance of glutathione followed by a gradual return to the control level in one hour (Figs 26 29 32) A trace of cystine was visible on the chromatogram of the 5 minute red cell sample (Fig 29) but this compound was not visible on the other chromatograms Little change took place in the amino acids of the leukocytes with possibly only a small decrease in the content of some of the constituents being found in the 5 minute sample (Fig 30)

The above findings have been confirmed and extended in *in vitro* experiments The latter studies have indicated that erythrocytes either do not degrade glutamine to glutamic acid and ammonia or do so only at a slow rate Both *in vivo* and *in vitro* studies showed that when the content of plasma glutamine is increased the quantity of glutathione is first depressed and then returns to normal levels without any notable change in glutamine concentration taking place This suggests that synthesis of glutathione by the red cell, which has recently been observed by other investigators (4 5 19) is accelerated by elevation of glutamine in plasma The major metabolic pathway for glutamine in the lymphocyte as judged from *in vitro* studies would appear to be the conversion to glutamic acid and ammonia with the subsequent formation of other amino acids from glutamic acid, probably by transamination It has been possible to observe reduction of glutathione content of lymphocytes incubated in plasma using a starting glutamine concentration of $5 \times 10^{3}$ M Glutathione was reduced to undetectable levels followed by a return toward normal during the course of the incubation From the above observations it would appear that the transpeptidation system in which glutamine participates in the degradation of glutathione is operative in the formed elements of the blood

cellular content of these compounds at the time the blood is drawn These findings led us to believe that the *glutamine glutathione trans* peptidation system described by Hanes *et al* (13) in kidney and pan




FIGS 19–24 Amino acid patterns in chronic lymphocytic leukemia from (19) 100 mg small lymphocytes (20) 0.5 ml packed red cells (21) 0 5 ml plasma and (22) 0.5 ml urine as compared to (23) normal plasma (0 5 ml ) and (24) normal urine (1 0 ml )



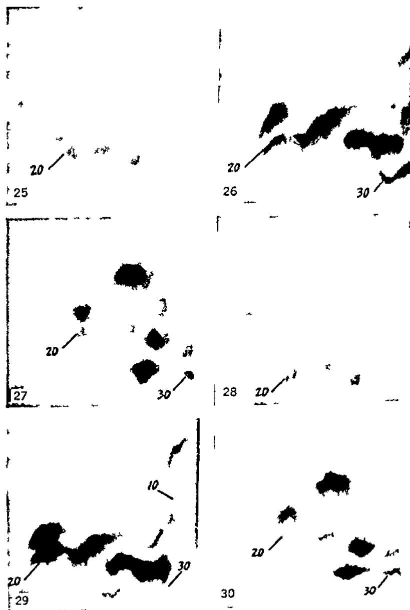

FIGS 25–33 Blood free amino acid changes after intraarterial glutamine injection (chronic lymphocytic leukemia) control samples (25-27) of plasma, red cells and small lymphocytes (28-30) plasma red cells and lymphocytes 5 minutes after injection (31-33) plasma, red cells and lymphocytes 60 minutes after injection. Extracts equivalent to 0.25 ml. plasma 1.0 ml. packed red cells and 100 mg lymphocytes used in each case

served as a result of alteration of extracellular concentrations of one or more of the amino acids When it is realized that these effects may also be dependent on nutritional hormonal, and other physiological factors the complexity of the situation becomes apparent and the danger of generalizing from a limited amount of data obtained under restricted conditions is emphasized. For these reasons the paper chromatographic technique is especially valuable for survey studies allowing a large number of samples to be examined for a variety of constituents in a relatively short period of time

These studies focus attention on the fact that in a high-count leukemia the metabolic activity of a large mass of blood cells may affect the amino

**Discussion**

The above observations serve to indicate a type of relationship which may exist between the amino acids of plasma and those of the formed elements of blood The results with glutamine show only one of probably many effects on metabolic reactions in the cells which may be ob



changes in free amino acids and glutathione may influence the production of red cells or their life span the finding of glutathione transpeptidase activity in the red cells may possibly explain certain findings which have been reported dealing with glutathione content of erythrocytes A number of amino acids can react with glutathione in the transpeptidase system and some e g methionine are even more active than glutamine (8 16) It is evident that glutathione content of erythrocytes may vary with changes in plasma amino acid levels Glutathione concentration in erythrocytes has been observed to be increased in hyperthyroidism after ACTH administration during protein starvation and in severe liver disease (15) This may be a result of the increased free amino acid levels in plasma observed in these conditions (6 7 9 14 24) Glutathione content is much higher in erythrocytes when rats are fed a diet deficient in methionine (11 18) This may be related to the fact that such diets generally result in a lowered level of free amino acids in plasma (3 26)

Summary

1 Investigation has been made of free or easily extractable amino acids in plasma, platelets leukocytes erythrocytes and urine in normal animals and man and in adult leukemic patients

2 Under a given set of circumstances each formed element of the blood appears to have a characteristic free amino acid pattern and differences occur in similar cell types in various species

3 An interrelationship of both plasma and cellular levels of free amino acids has been found, and it is emphasized that careful comparison of amino acid distribution in cells plasma and urine is necessary in order properly to evaluate any changes observed

4 Low levels of glutamine have been observed in the plasma of a number of the leukemic patients studied Some of the relations of this finding to the glutamine and glutathione content and metabolism of the cellular constituents of blood have been explored

REFERENCES

1 Christensen, H N Riggs T R Fischer H and Palatine J M *J Biol Chem* 198 17 (1952)
2 Christensen, H N Streicher J A and Elbinger R L *J Biol Chem* 172 515 (1948)
3 Denton A E Williams J N Jr and Elvehjem C A *J Biol Chem* 186 377 (1950)
4 Dimant, E Landsberg, F and London, I M *J Biol Chem* 213 769 (1955)
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6 Flock, E V Block M A Grindlay J H Mann F C and Bollman J L *J Biol Chem* 200 529 (1953)

acid content of the blood plasma to a considerable extent and thus influence the amino acid metabolism of the organism as a whole in a specific manner depending on the predominating cell type In order to distinguish those changes attributable to the blood cells and those arising from abnormalities in other body tissues or from induced nutritional abnormalities it is necessary to study an adequate number of patients maintained under carefully controlled conditions The impression obtained must then be confirmed by employing various metabolites in elevated concentration both *in vivo* and *in vitro* As a corollary it is imperative to inactivate the enzymes in the blood samples as rapidly as possible prior to analysis since leukocytes can continue to metabolize various substances *in vitro* at a rapid rate Numerous studies performed in this laboratory have shown that the procedures employed have reduced artifactual changes in amino acid content to a minimum

The fact that morphologically distinct cell types generally have a characteristic free amino acid pattern and probably metabolize amino acids in different ways and at different rates make it apparent that in order to determine whether a cell type from a patient with leukemia is different from normal comparison with the corresponding normal cell type should always be made where possible In the instance in which a comparison was made of neutrophilic polymorphonuclear leukocytes from patients with granulocytic leukemia and reticulum cell sarcoma and from a normal individual it was felt that differences observed could be explained as well by assuming a metabolic disturbance resulting indirectly from the disease as by attributing the changes to abnormalities in the cells themselves Our observations have led us to conclude that the changes in plasma and urine amino acids in patients with chronic lymphocytic leukemia can be attributed at least to a certain extent to the metabolism of the lymphocytes

Roberts *et al* (20 22) have demonstrated that free glutamine is low or absent in solid tumors in animals and in both ascites tumor cells and ascitic fluid These investigators have also demonstrated the rapid utilization of glutamine by ascites tumor cells and its conversion to glutamic acid White *et al* (25) have shown that glutamic acid may be elevated and glutamine somewhat depressed in the plasma of rats with transplantable tumors Our observations make it seem probable that in many human leukemias there is also rapid utilization of glutamine and a consequent elevation of glutamic acid Elevation of plasma alanine and aspartic acid content has also been observed

The problem of anemia in the leukemias makes important a study of the chemistry of red cells Although we do not know whether the

changes in free amino acids and glutathione may influence the production of red cells or their life span the finding of glutathione transpeptidase activity in the red cells may possibly explain certain findings which have been reported dealing with glutathione content of erythrocytes A number of amino acids can react with glutathione in the transpeptidase system and some e g methionine are even more active than glutamine (8 16) It is evident that glutathione content of erythrocytes may vary with changes in plasma amino acid levels Glutathione concentration in erythrocytes has been observed to be increased in hyperthyroidism after ACTH administration during protein starvation and in severe liver disease (15) This may be a result of the increased free amino acid levels in plasma observed in these conditions (6 7 9 14, 24) Glutathione content is much higher in erythrocytes when rats are fed a diet deficient in methionine (11 18) This may be related to the fact that such diets generally result in a lowered level of free amino acids in plasma (3 26)

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1 Christensen H N Riggs T R Fischer H and Palatine I M *J Biol Chem* 198 17 (1952)
2 Christensen H N Streicher J A and Elbinger R L *J Biol Chem* 172 515 (1948)
3 Denton, A E Williams J N Jr and Elvehjem C A *J Biol Chem* 186 377 (1950)
4 Dimant E Landsberg, E and London I M *J Biol Chem* 213 769 (1955)
5 Elder H A and Mortensen R A *J Biol Chem* 218 261 (1956)
6 Flock, E V Block M A Grindlay J H Mann, F C and Bollman J L *J Biol Chem* 200 529 (1953)

7 Flock E V Mann F C and Bollman J L *J Biol Chem* 192 293 (1951)
8 Fodor P J Miller A and Waelsch H *J Biol Chem* 202 551 (1953)
9 Friedberg F and Greenberg D M *J Biol Chem* 168 405 (1947)
10 Friedberg F and Greenberg D M *J Biol Chem* 168 411 (1947)
11 Grunert R R and Philips P H *Proc Soc Exptl Biol Med* 76 642 (1951)
12 Handler P Kamin H and Harris J S *J Biol Chem* 179 283 (1949)
13 Hanes C S Hird F J R and Isherwood F A *Nature* 166 288 (1950)
14 Henderson L M Schurr P E and Elvehjem C A *J Biol Chem* 177 815 (1949)
15 Henneman D H Altschule M D and Goncz R M in Glutathione A Symposium (S P Colowick A Lazarow E Racker D R Schwartz E Stadtman H Waelsch eds ) p 299 Academic Press 1954
16 Hird F J R and Springell P H *Biochem J* 56 417 (1954)
17 Johnson C A and Bergeim O *J Biol Chem* 188 833 (1951)
18 Mortensen R A *J Biol Chem* 204 239 (1953)
19 Mortensen R A Haley M I and Elder H A *J Biol Chem* 218 269 (1956)
20 Roberts E and Borges P R F *Cancer Research* 15 697 (1955)
21 Roberts E and Frankel S *Cancer Research* 9 645 (1949)
22 Roberts E and Tanaka T *Cancer Research* 16 204 (1956)
23 Rouser G Thesis University of Rochester 1952
24 Walshe J M *Quart J Med* [n s ] 22 483 (1953)
25 White J M Ozawa G Ross G A L and McHenry E W *Cancer Research* 14 508 (1954)
26 Wu C *J Biol Chem* 207 775 (1954)

## General Discussion

Dr Milton Toporek (Ann Arbor Michigan) I should like to comment on Dr Waisman s observations

We have run numerous electrophoretic patterns on sera and plasma of leukemic patients and an almost invariable finding is that the globulins tend to be elevated with respect to normal and the albumin is somewhat decreased This has been observed by many other people Also the same sort of situation has been observed in almost any acute or chronic situation that one can think of

I believe the amino acid picture—in other words the change from normal—which Dr Waisman has observed is directly related to this sort of situation Furthermore I believe the fact that no significant change was observed as a result of therapy is merely a reflection of the fact that the primary condition which leads to the aberration of plasma protein synthesis has simply not been touched by the therapy

Dr Ralph Engle (New York New York) We have been interested in the amino acid excretion in the urine of patients with multiple myeloma a disease closely related to leukemia (1 3)

Using the two dimensional paper chromatography technique as outlined by Dent to study the urines in twenty six patients with multiple myeloma we have found that twenty five have had essentially normal excretions of amino acids One patient however who has proved to be extremely interesting had a very large excretion of all amino acids On further study this particular patient was found to have the complete adult Fanconi syndrome that is the patient was excreting large quantities of glucose uric acid bicarbonate and a number of other substances into the urine which ordinarily would be held back in the plasma

Finally the most interesting feature about this patient with the Fanconi syndrome was that at autopsy his kidney tubules were almost completely destroyed by crystalline material which had precipitated out in the epithelial cells of the renal tubule not in the lumen We feel that this marked destruction of the renal tubular epithelium accounted for the adult Fanconi syndrome in this patient with multiple myeloma The nature of the crystalline material is not known with certainty The patient excreted up to 50 g of Bence Jones protein into the urine per day however and this may have become deposited in the cells

REFERENCES

1 Engle R L Jr J Mount Sinai Hosp 23 193 (1956)
2 Engle R L Jr and Wallis L A Am J Med accepted for publication
3 Wallis L A and Engle R L Jr Am J Med accepted for publication

# 22

## Metabolic Stability of PNA and DNA in Human Leukemic Leukocytes, The Function of Lymphocytes[1]

L D HAMILTON

Divisions of Experimental and Clinical Chemotherapy
The Sloan Kettering Institute for Cancer Research New York New York

*Introduction*

The key role of the nucleic acids in the etiology and pathophysiology of the leukemias has been recognized in this International Symposium there are sessions devoted to genetic factors in the transmission of leukemia and to nucleic acids as the target for the chemotherapy of leukemia

There are two main types of nucleic acid pentosenucleic acid (PNA) and deoxyribonucleic acid (DNA) (The nucleic acids and their precursors are being so intensively investigated that workers are too harried to refer to these substances by their full proper names instead they use agglutinations of capital letters see Buchanan and Goldthwait, this Symposium ) DNA is found in all living cells with the exception of some viruses which contain its close relative PNA In mammalian cells 90% of PNA is in the cytoplasm and 10% in the nucleus in plants and animals all the DNA is in the nucleus localized in the chromosomes—the gene carriers (Fig. 1)

A wealth of circumstantial evidence from classical cytology and genetics all pointed to the nucleic acids as the best candidates for controllers of the character and rate of growth of cells The first direct evidence that nucleic acids were themselves the actual genetic material came with the isolation from bacteria of DNA that could confer different morphological (1) or enzymic capacities on other bacteria of the

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[1] The work has been aided by grants from the National Cancer Institute of the United States Public Health Service C 1813 and from the Commonwealth Fund.

[2] Scholar of the American Cancer Society

same species and endow those bacteria and their descendents with these properties (18 33) For example it is possible to transform a penicillin sensitive pneumococcus into a penicillin resistant organism by incubating the sensitive organism with DNA isolated from the resistant strain The fact that almost pure DNA effects this bacterial transformation is the most convincing evidence that DNA is the hereditary material (32 33) Complementary evidence of a genetic role for DNA comes from the observation that a bacteriophage injects about $2 \times 10^{10}$ γ of DNA and perhaps one tenth as much protein into a bacterium and it is this DNA that appears capable of self replication in the bacterial cell with the production of numerous new phages (29 30 31) The recent demonstration that only the PNA of tobacco mosaic virus is infective points to an analogous genetic role for PNA in plant viruses (20)

Although similar direct evidence is not yet forthcoming in mammals quantitative cytochemical and genetic evidence supports the genetic significance of DNA other circumstantial and controversial evidence has linked PNA to protein synthesis Since most evidence indicates that cancer cells result from modifications of the genetic equipment of normal cells it seems reasonable to hope that differences between cancer and normal cells might be demonstrable in their nucleic acids Accordingly studies are being made on the biosynthesis metabolism and structure of the nucleic acids of normal and leukemic human leukocytes

## Biosynthesis of Nucleic Acids

Mammals do not have a nutritional requirement for preformed nucleic acids or for any nucleic acid component, other than phosphate they are able to synthesize their complex polynucleotides from simple precursors It is possible therefore that the utilization of larger building blocks for nucleic acid synthesis such as exogenous purines is in effect a by passing of part of the normal biosynthetic pathway It has been shown that a wide variety of organisms can utilize exogenous purines for the synthesis of polynucleotides (6) Exogenous adenine is not only utilized as such in polynucleotides but is also transformed into polynucleotide guanine similarly exogenous guanine is utilized as such and transformed in a number of species into polynucleotide adenine There is in fact a considerable diversity in patterns of purine utilization (6 22) A much simplified diagram of the pathways of purine incorporation and interconversion is given in Fig 2

## Utilization of the Purines in Man

This communication describes experiments on the utilization of $C^{14}$ labeled purines for nucleic acid synthesis in the leukocytes of patients

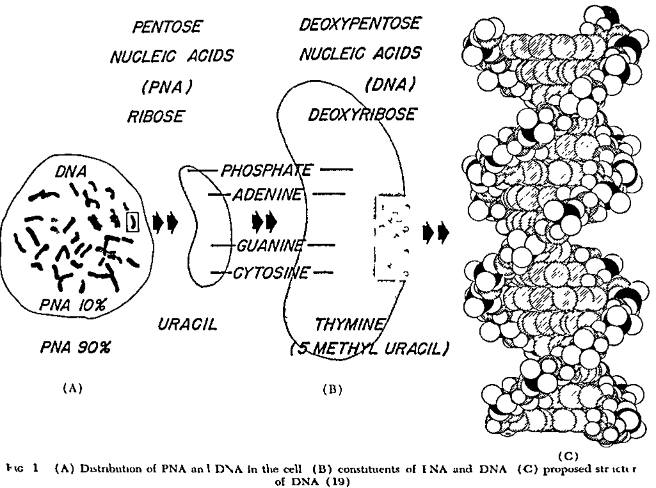



FIG 1 (A) Distribution of PNA and DNA in the cell (B) constituents of PNA and DNA (C) proposed structure of DNA (19)

eral blood Although the total white count fluctuated considerably ranging *between 80 000 and 200 000/mm³ the intensity of radioactivity of* the purine in the nucleic acids remained constant This is consistent with the idea that leukocytes in the peripheral blood of a patient with leukemia are a random sample of the total leukocytes of that individual and that the leukocytes in the peripheral blood represent only a propor

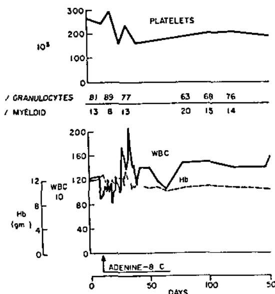



FIG 3 M W male patient, age 30 having chronic granulocytic leukemia

tion of the total leukocytes in the body Similar fluctuations are seen (Fig 4) in the lymphocyte count on F R a male patient, age 47 having chronic lymphatic leukemia for whom representative data on the incorporation of adenine into nucleic acids will be given Although his peripheral count ranged from 60 000 to over 250 000/mm³ the curves of the amount of radioactivity in purines of nucleic acids of lymphocytes plotted against time remained unchanged Again this suggests that lymphocytes in the peripheral blood are a random sample of total lymphocytes of that individual and also that they represent only a small proportion of the total lymphocytes of the body This patient during the period of study had at least 90% mature small lymphocytes in his peripheral blood

Blood was collected at intervals after the single injection of the labeled

having chronic leukemia The patterns of purine utilization in man are unknown except for some preliminary observations (21 23 24) Since patients having chronic leukemia represent a store of readily accessible cells which have an intensively active nucleic acid synthesis studies on the utilization of $C^{14}$ labeled purines for nucleic acid synthesis were undertaken in such subjects For this purpose patients having chronic lymphatic or chronic granulocytic leukemia with leukocyte counts of at least 100 000/mm ³ a reasonably good hemoglobin and in good general

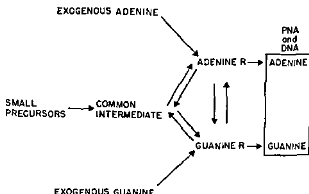



FIG 2 Possible pathways of purine incorporation and interconversion into PNA and DNA

condition were selected In the lymphatic leukemia patients the pre dominant cell type was the small lymphocyte and in the granulocytic patients the leukocytes were polymorphonuclears Adenine 8 $C^{14}$ (200 $\mu$c ) was given once intravenously to two patients having chronic lym phatic and to two having chronic granulocytic leukemia another patient having chronic lymphatic leukemia was given guanine 8 $C^{14}$ A patient with a leukemoid reaction consisting of mature polymorphonuclear leukocytes 50 000 to 70 000/mm ³ was given adenine 8 $C^{14}$ This patient had a bronchogenic carcinoma with direct invasion of the ribs the leukemoid reaction was presumed to be secondary to bone marrow ir ritation as the result of this local invasion No infection or other ex planation could be assigned for the elevated leukocyte count

Figure 3 shows the main hematological findings in M W a male patient age 30 having chronic granulocytic leukemia for whom rep resentative data on incorporation of adenine into nucleic acids will be given It will be noted that during the period of study the proportion of mature polymorphonuclear leukocytes together with the myelocytes and metamyelocytes made up usually at least 90% of the cells in the periph

Figure 5 shows the incorporation of adenine into the PNA of the granulocytes of patient M W The data are plotted uniformly the ordinate radioactivity of the purines in counts per minutes per micro mole isolated from the PNA of granulocytes the abscissa time in days

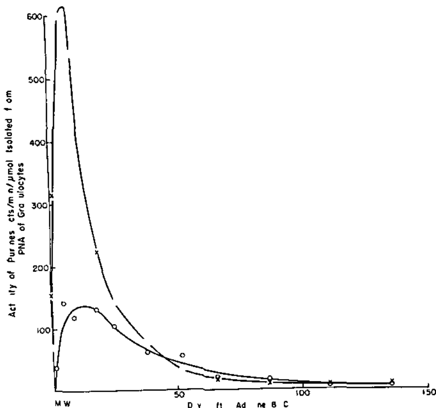



FIG 5 Incorporation of adenine 8-C[14] into PNA of granulocytes of patient M W having chronic granulocytic leukemia

after the single intravenous injection of labeled purine It is seen that in granulocytes there is extensive incorporation of adenine as such into PNA and eventual transformation of adenine into PNA guanine there was little significant radioactivity demonstrable after 100 days The in corporation of adenine and the metabolic activity of the PNA of the granulocytes is much like that described for PNA in a wide variety of tissues Similar data are plotted in Fig 6 which show the incorporation

purine Leukocytes were separated from red cells by the use of dextran and standard procedures were employed for the separation of pentose *nucleic acid and deoxyribonucleic* acid from the leukocytes The radio activities were determined of the separated individual purines of the nucleic acids of the leukocytes from the first two patients studied the purines were separated by paper chromatography The radioactivities

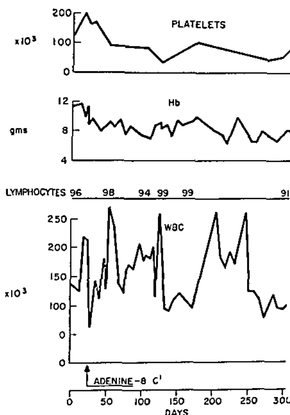



Fig 4 F R male patient age 47 having chronic lymphatic leukemia

of the PNA nucleotides and of the DNA purines and pyrimidines of nucleic acids of the leukocytes were determined on later patients PNA nucleotides were separated by paper electrophoresis and the DNA bases by paper chromatography The leukocytes of the first two patients were extracted with trichloracetic acid to remove free purines and acid soluble nucleosides and nucleotides Leukocytes of later patients were extracted with perchloric acid these perchloric acid extracts were neu tralized and frozen for later analysis of the acid soluble components

of the nucleus and PNA of the cytoplasm—and it is probable that there are several slightly different PNAs in the cytoplasm It has also been suggested that DNA may be metabolically heterogeneous (3) evidence for heterogeneity has come from fractionation of DNA (2 7 8 17) In the meanwhile a considerable literature has accumulated on the relative rates of PNA and DNA turnover (6 46) From these studies it would appear that DNA turnover is as rapid as that in PNA at least in bone marrow and intestine—that is in rapidly dividing tissues It may be deduced therefore from the fact that the turnovers of the PNA and DNA of leukemic granulocytes are of the same order of intensity that these cells are rapidly dividing

The data obtained on the incorporation of adenine into the nucleic acids of lymphocytes sharply contrast with those obtained from granulocytes Figure 7 is a plot of the incorporation of adenine into the PNA of lymphocytes it is seen that as in the granulocytes adenine is incorporated as such into PNA adenine and transformed into PNA guanine The extent of this incorporation and transformation into the PNA of the lymphocyte is very similar to that seen in the PNA of the granulocytes In contrast with the PNA of the granulocyte there is prolonged retention of radioactivity in the PNA purines of the lymphocyte Thus significant radioactivity could still be found at 280 days after the injection of labeled adenine in both PNA adenine and PNA guanine It can be seen that the declines in the radioactivity of PNA purines when both purines are equally labeled lie parallel The incorporation of adenine into the DNA of lymphocytes (Fig 8) is much less extensive than that seen in PNA Again adenine is incorporated as such and transformed into DNA guanine As in the PNA there is prolonged retention of radioactivity in DNA adenine and in DNA guanine the declines being parallel in both purines after both are equally radioactive The lesser incorporation into DNA as compared to PNA is similar to the relationship of DNA to PNA seen in slowly dividing tissues such as resting liver

As seen in Figs 7 and 8 considerable variation in the ratio of incorporation of PNA to that of DNA can be observed depending on the time of sampling after the administration of the isotope Such variations in the time relations of experiments are the cause of much confusion in the literature on the relative ratio of PNA to DNA labeling If as it now seems reasonable to assume PNA turnover in the cell is related to protein synthesis or some closely allied metabolic function and DNA turnover to cellular division and multiplication the turnover of PNA would be relatively greater than that of DNA in tissues that were actively engaged in synthetic processes but were themselves only slowly divid

of adenine into the DNA of the same granulocytes It is apparent that the incorporation and transformation of adenine is similar to that seen in PNA and that the extent of the incorporation and transformation and the retention of isotope in the DNA adenine and the DNA guanine are very similar to that in the PNA

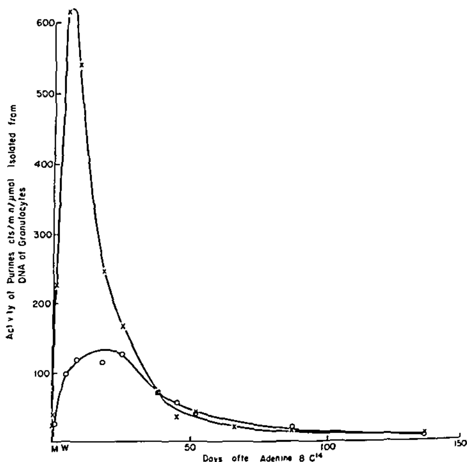



*FIG 6 Incorporation of adenine 8-$C^{14}$ into DNA of granulocytes of patient M W having chronic granulocytic leukemia*

There has been much said in the literature about the relative incorporation of different precursors into PNA and DNA (6) Much of the discussion has been based on experiments in which only one or a few points were plotted against time after the administration of the isotope Further it must be recognized that PNA is heterogeneous metabolically (46 47) There are at least two main metabolic classes of PNA–PNA

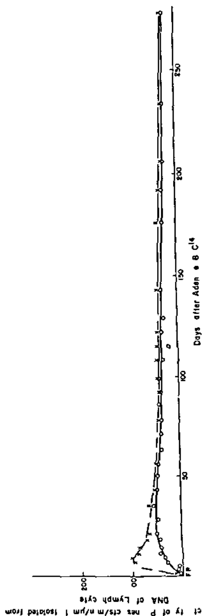



FIG 8 Incorporation of adenine 8 $C^{14}$ into DNA of lymphocytes of patient F R having chronic lymphatic leukemia

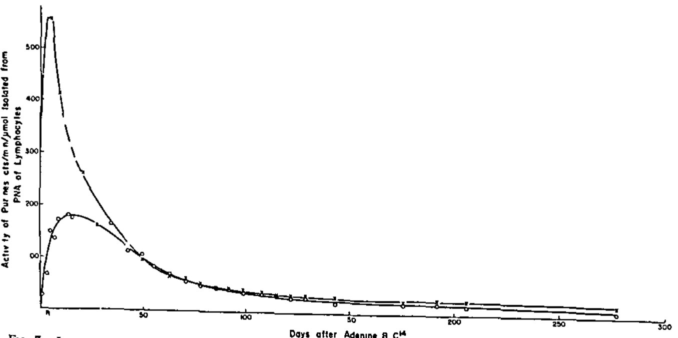



Fig 7 Incorporation of adenine-8-C14 into PNA of lymphocytes of patient F R having chronic lymphatic leukemia

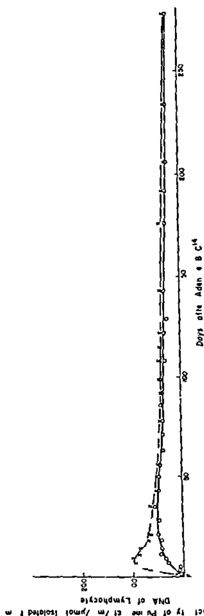



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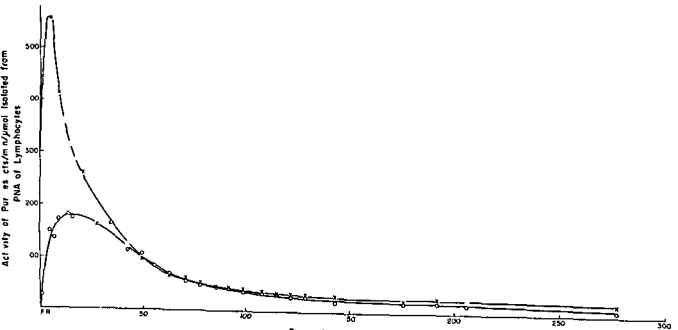



FIG 7 Incorporation of adenine 8-$C^{14}$ into PNA of lymphocytes of patient F R having chronic lymphatic leukemia

periods and others surviving for longer The situation for the lymphocyte DNA data is slightly different as indicated by the prolonged retention of isotope If the log of radioactivity is plotted against linear time it is seen that the decline in radioactivity in lymphocyte DNA and indeed in lymphocyte PNA can be divided into two phases an initial rapid decline followed by a slow decline having a half time of the order of 300 days It has already been made clear from the ratio of PNA turnover to DNA turnover that the lymphocyte is a more slowly dividing cell than the granulocyte it is reasonable to assume therefore that the leukemic lymphocyte survives longer than does the leukemic granulocyte Corresponding calculations on the DNA of the lymphocytes before the slow decline in the radioactivities of the purines show that leukemic lymphocytes survive on the average for 85 days

It is conceivable that the slow decline which has a half time of the order of 300 days represents a second type of leukemic lymphocyte with a longer life than 85 days An alternative interpretation is that this slow decline represents the reutilization by lymphocytes of large fragments of the nucleic acids or nucleoproteins of their progenitors (cf 25 30a) The present biochemical evidence does not permit decision whether this prolonged retention of isotope reflects the survival of some cells for a long time or such a reutilization

It may be postulated that this reutilization if it occurs must be a reutilization of large fragments of nucleic acids or nucleoproteins First, *the decline in radioactivity of DNA adenine is parallel to that in DNA guanine* This may mean that a portion of polynucleotide large enough to contain both adenine and guanine had to be reutilized To harmonize with current ideas of the DNA macromolecule (19 50) such a fragment must contain at least four nucleotides Second there is additional evidence that reutilization of free purines does not take place (14) Furthermore the initial rapid incorporation of exogenous purines suggests that the sharp break in the slope of the radioactivity of the DNA purines of the lymphocytes must be due to a metabolic process distinct from that associated with the initial incorporation of free purine

Other kinds of evidence speak in favor of such a reutilization The phagocytosis of lymphocytes by reticulum cells in lymph nodes and the differentiation of these reticulum cells into new lymphocytes have been repeatedly observed (5 27) Maximow noted and then ignored this differentiation of lymphocytes from reticulum cells (38) More recently cells have been described that are intermediate between these reticulum cells and large lymphocytes which still contain pyknotic nuclei in their cytoplasm such observations have been made by means of both the

ing The turnover of DNA could be as great as that of PNA in tissues which were dividing rapidly as well as synthesizing rapidly or alternatively in cells which were dividing more slowly but were less active metabolically The granulocytes are a good example of a tissue in which PNA and DNA turnovers appear to be almost identical this is therefore a cell that is assumed to be dividing rapidly In contrast lymphocytes appear to be a much more slowly dividing tissue The two types of leukemic white cells thus form a neat contrast of cell types in one DNA turnover is as great as PNA turnover in the other DNA turnover is much less than PNA turnover Although as a general rule the metabolic stability of DNA is much greater than that of PNA it now seems that in rapidly dividing tissues the metabolic stability of DNA is not very different from that of PNA

## Metabolic Stability of DNA and Survival Time of Leukocytes

There is evidence already briefly referred to to the effect that incorporation of precursors into DNA is directly proportional to the mitotic activity of the particular tissue so that turnover is greatest in rapidly dividing tissues such as intestine tumors and regenerating liver and that in many tissues when the labeled compounds are incorporated they disappear from the cellular DNA far more slowly than from PNA This does not apply to granulocytes or lymphocytes In granulocytes the decline in radioactivity of PNA is the same as that of DNA and in lymphocytes the declines in radioactivities of the PNA purines only slightly exceed those of the DNA purines There is no coherent evidence that there is metabolic turnover of DNA during the life of the cell independent of cellular division This is not surprising If DNA is indeed *the* genetic material it would seem unlikely that it would break down before or during cell division else it would be an impossibly unstable transmitter of inheritance (34)

If the assumption is made that DNA once formed in a cell persists essentially unchanged for the life of the cell the average survival time of that cell can be calculated from the DNA turnover data Analyses of the DNA data by the solution of the Volterra integral equation of the first kind on the assumption that the DNA remains with the cell for its life give an average survival time for the leukemic granulocyte of 23 days "Average survival time" means the time from the earliest formation of the cell in the bone marrow to the termination of its life in the peripheral blood that is it includes both formation time and intravascular life span It must be emphasized that 23 days is an approximate figure—it represents the average of cells surviving for shorter

periods and others surviving for longer The situation for the lympho cyte DNA data is slightly different as indicated by the prolonged reten tion of isotope If the log of radioactivity is plotted against linear time it is seen that the decline in radioactivity in lymphocyte DNA and in deed in lymphocyte PNA can be divided into two phases an initial rapid decline followed by a slow decline having a half time of the order of 300 days It has already been made clear from the ratio of PNA turnover to DNA turnover that the lymphocyte is a more slowly divid ing cell than the granulocyte it is reasonable to assume therefore that the leukemic lymphocyte survives longer than does the leukemic granulocyte Corresponding calculations on the DNA of the lympho cytes before the slow decline in the radioactivities of the purines show that leukemic lymphocytes survive on the average for 85 days

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light (Trowell personal communication) and electron microscope (Dempsey personal communication) These histological observations provide morphological grounds for thinking that there may be utilization of large fragments of lymphocyte nucleic acids or nucleoproteins mediated by phagocytosis

Whatever the interpretation of the data their consistency is clear For example preliminary data on the incorporation of guanine-8 $C^{14}$ into the DNA purines of another patient S B a female age 64 having chronic lymphatic leukemia is charted in Fig 9 It can be readily seen that the results are very similar to those obtained with adenine the chief difference being that guanine is incorporated as such into the DNA more extensively than it is transformed into DNA adenine The upper slope represents DNA guanine and the lower DNA adenine The data obtained for PNA nucleotides were similar incorporation into guanylic acid exceeding transformation into adenylic acid Both PNA and DNA showed prolonged retention of isotope in the PNA nucleotides and the DNA purines retention in the DNA exceeded that in the PNA Thus results obtained with exogenous guanine paralleled those with exogenous adenine Further experiments are in progress in which $C^{14}$ labeled adenine and orotic acid and guanine and orotic acid are given conjointly in the hope that something more definite can be learned about the meaning of prolonged retention of isotope in lymphocytes

### Work of Other Investigators on the Survival Time of Leukocytes

Many investigators have tried by a variety of techniques to estimate the average survival time of normal and leukemic leukocytes This discussion is confined to those who have used DNA turnover as an index Osgood *et al* (42) measured the incorporation of radioactive phosphorus into the DNA of different types of leukocytes In their first publication they tried to deduce life span solely from the uptake of $P^{32}$ into the DNA i e no data were given on the retention of isotope in the DNA They claimed that the granulocyte in chronic granulocytic leukemia had a life span of 3 days how this was deduced was obscure Similarly they stated the life span of the lymphocyte in chronic lymphocytic leukemia to be 30 days [3]

[3] After this presentation my attention was drawn to a later publication by Osgood *et al* (43) in which their early data were recalculated The mathematical assumptions underlying these calculations are not explicit and evaluation must therefore await full publication Recalculation of their data gave an average survival time of 5 days for the granulocyte in chronic granulocytic leukemia and 84 days for the lymphocyte in chronic lymphatic leukemia the latter figure agrees with that found in the present study

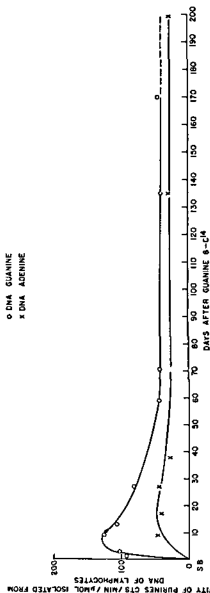



FIG 9 Incorporation of guanine 8 $C^{14}$ into DNA of lymphocytes of patient S B having chronic lymphatic leukemia

Kline and Cliffton (36) estimated the average survival time of leukocytes in normal subjects by charting the incorporation and turnover of $P^{32}$ in the DNA of total leukocytes they arrived at 13 2 days as the average for all cell types They also used a solution of the Volterra integral equation for their calculations of the average survival time this procedure is analogous to that used by Shemin and Rittenberg for the life span of the red cells (45)

Ottesen (44) applied an ingenious technique for the separation of granulocytes and lymphocytes He then studied the incorporation of $P^{32}$ into the DNA of both cell types in an 88 year old woman and a 44 year old woman both were hematologically normal From other mathematical considerations he derived a method of calculation not much different from the method of Shemin and Rittenberg The DNA data by his solution gave an average survival time of the granulocyte of 9 days his lymphocyte data appear much less susceptible to satisfactory mathematical analyses of this type He suggests that lymphocytes fall into two groups one group with an average survival time of 3 to 4 days and another with an average survival time of 100 to 200 days In his older patient 22% of lymphocytes were in the short lived group and 78% in long lived group in the younger patient the short lived cells formed 11% and the long lived 89% of the total lymphocytes in circulation

Preliminary study of the data obtained by us with $C^{14}$ adenine on a patient with a leukemoid reaction in which the predominant cell was the polymorphonuclear leukocyte gave as a first approximation an average survival time for the granulocyte of 9 days This is in good agreement with the findings of Ottesen for the normal granulocyte If the figure of 9 days is accepted as the average survival time of the normal granulocyte and the average survival time of leukocytes of both cell types is 13 days the normal lymphocyte must clearly have a survival time of longer than 13 days By rough calculation with lymphocytes assumed to constitute one third of the leukocytes the average survival time must be at least 21 days The results therefore suggest that both leukemic granulocytes and lymphocytes survive longer in chronic leukemic patients than do normal granulocytes and lymphocytes in the nonleukemic subjects

### The Function of the Lymphocyte

The function of the lymphocyte has long been the subject of speculation and controversy (16 49) The high nucleic content of the lymphocyte (the mature lymphocyte is virtually a naked nucleus) and the

possibility from morphological and biochemical evidence that there may be reutilization of lymphocytes by phagocytosis invite speculation on how their nucleic acids are related to function If the main function of the nucleic acids is to act as genetic codes for the construction of different proteins it would be reasonable to expect that their presence in such large amounts in lymphocytes denotes a similar function in lymphocytes Awareness that lymphatic tissue is an important site of protein synthesis—as indicated by antibody production—has been obscured by an inability to differentiate between the roles of lymph nodes and of the mature lymphocyte (26) Clarity was not aided by the partisanship of supporters of one or another cell type as the main carrier of antibodies

The presence of such large amounts of nucleic acid and this process of reutilization suggest that lymphocytes may function mainly as a store of nucleic acids These nucleic acids in the lymphocytes would serve as templates for the synthesis of the protein antibodies Clearly there would be numerous nucleic acids each capable of the production of a specific antibody This hypothesis provides an explanation of the ability to transfer immune reactions by the transfer of lymph nodes or lymphocytes for example Chase succeeded in transferring hypersensitivity to tuberculin by means of cells from lymph nodes of tuberculous guinea pigs (9) This observation has been confirmed by many workers and extended by Chase (10 11 13 35 37 39 48) Mitchison obtained cells from lymph nodes regional to tumor homografts and transferred them either minced or in suspension to a secondary host the transferred cells conferred immunity on the recipient (40 41) Similar experiments have been performed by Billingham *et al* (4) on skin transplantation between different lines of mice Since antibodies are incapable of self replication (28) and there is clear evidence that not enough passive antibody could be transferred to account for the long continued immunity conferred on the recipient (12 15) it is likely that some self replicating mechanism was carried over in this way If we accept the function of the lymphocytes as a store of specific nucleic acid for the production of specific antibodies along with the process of reutilization by phagocytosis of large fragments of these nucleic acids these observations can be readily explained Similarly the ability to prevent the production of antibody in later life by exposing embryos to specific antigens (4) can be explained by the exhaustion of the specific DNA s responsible for producing the homologous antibodies this disappearance of DNA may be due to an absence in the embryo of the conservation mechanism represented by phagocytosis and reutilization

The continued formation of antibody long after limited contact be

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15 Dixon, F J Roberts J C and Weigle W O *Federation Proc* (in press)
16 Drinker C V and Yoffey J M in Lymphocytes Lymph and Lymphoid Tissue Their Physiological and Chemical Significance Harvard University Press Cambridge 1941
17 Elson D Gustafson T and Chargaff L *J Biol Chem* 209 285 (1955)
18 Ephrussi Taylor H., *Cold Spring Harbor Symposia Quant Biol* 16 445 (1951)
19 Feughelman M Langridge R Seeds W E Stokes A R Wilson H R Hooper C W Wilkins M F H Barclay R K and Hamilton L D *Nature* 175 834 (1955)
20 Gierer A and Schramm G *Nature* 177 702 (1956)
21 Hamilton L D *Nature* 172 457 (1953)
22 Hamilton L D *Ann N Y Acad Sci* 56 961 (1953)
23 Hamilton L D *J Clin Invest* 33 939 (1954)
24 Hamilton L D *J Clin Invest* 34 938 (1955)
25 Hamilton L D *Ca Bull Cancer Progr* 5 159 (1955)
26 Harris T N and Harris S *Am J Med* 20 114 (1956)
27 Heiberg, K A *Acta Med Scand* 65 443 (1926–27)
28 Heidelberger M Treffers H P Schoenheimer R Ratner S and Rittenberg, D *J Biol Chem* 144 555 (1942)
29 Hershey A D *Virology* 1 108 (1955)
30 Hershey A D in Enymes Units of Biological Structure and Function (Oliver H Gaebler ed ) Academic Press New York 1956
30a Hershey A D and Burgi E *Cold Spring Harbor Symposia Quant Biol* 21 in press
31 Hershey A D and Chase M *J Gen Psysiol* 36 777 (1953)
32 Hotchkiss R D in Unites biologiques doues de continuite genetique Éditions du Centre National de la Recherche Scientifique Paris 1949
33 Hotchkiss R D *Harvey Lectures* 49 124 (1954)
34 Hotchkiss R D in "The Nucleic Acids" (E Chargaff and J N Davidson eds ) Vol 2 Academic Press New York 1955
35 Kirchheimer W and Weiser R S *Proc Soc Exptl Biol Med* 66 166 (1947)
36 Kline D L and Cliffton E E *J Appl Physiol* 5 79 (1952)
37 Lawrence H S *Proc Soc Exptl Biol Med* 71 516 (1949)
38 Maximow A A cited by Bloom W in Handbook of Hematology Vol 2 Paul B Hoeber New York 1938
39 Metaxas M N and Metaxas Buehler M *Proc Soc Exptl Biol Med* 69 163 (1948)
40 Mitchison N A *Nature* 171 267 (1953)
41 Mitchison N A *J Exptl Med* 102 157 (1955)
42 Osgood E E Tivey H Davison K B Seaman A J and Li J G *Cancer* 5 331 (1952)
43 Osgood E E Seaman A J Tivey H and Rigas D A *Rev hématol* 9 543 (1954)
44 Ottesen J *Acta Physiol Scand* 32 75 (1954)
45 Shemin D and Rittenberg, D *J Biol Chem* 166 627 (1946)
46 Smellie R M S in The Nucleic Acids (E Chargaff and J N Davidson eds ) Vol 2 Academic Pres New York 1955

tween tissues and antigen in the adult is readily understood if phagocytosis and reutilization of nucleic acid are invoked this allows a continued and selective preservation of templates necessary for the production of specific antibodies It is postulated that the embryo has an enormous variety of species of nucleic acids capable of producing antibodies to most proteins Exposure of the embryo to antigen might lead to breeding out of the specific nucleic acids responsible for producing the antibody to that antigen In the adult potentialities not bred out in the embryo remain the presence of new antigen selectively stimulates the production of nucleic acids responsible for the synthesis of specific antibody and the process of phagocytosis with reutilization of nucleic acids allows for their selective preservation

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REFERENCES

1 Avery O T MacLeod C and McCarty M *J Exptl Med* 79 137 (1944)
2 Bendich, A Fresco J R Rosenkranz H S and Beiser S M *J Am Chem Soc* 77 3671 (1955)
3 Bendich A Russell P J Jr and Brown G B *J Biol Chem* 203 305 (1953)
4 Billingham R E Brent L and Medawar P B *Nature* 172 603 (1953)
5 Bloom W *in* Handbook of Hematology " Vol 2 Paul B Hoeber New York 1938
6 Brown G B and Roll P M *in* "The Nucleic Acids" (E Chargaff and J N Davidson eds ) Vol 2 Academic Press New York 1955
7 Brown G L and Watson M *Nature* 172 339 (1953)
8 Chargaff E Crampton C F and Lipshitz R *Nature* 172 289 (1953)
9 Chase M W *Proc Soc Exptl Biol Med* 59 134 (1945)
10 Chase M W *Federation Proc* 10 404 (1951)
11 Chase M W *in* "The Nature and Significance of the Antibody Response" Columbia University Press New York 1953
12 Chase M W and Wager O A *Federation Proc* in press
13 Cummings M M Hoyt M and Gottshall R Y *Public Health Repts (U S )* 62 994 (1947)
14 Dancis J and Balis M E *J Biol Chem* 207 367 (1954)

# 23

## Urinary Uric Acid Excretion in Leukemia[1]

IRWIN H. KRAKOFF

*Chemotherapy Service, Memorial Center*
*and the Division of Clinical Chemotherapy*
*Sloan-Kettering Institute, New York, New York*

Serum uric acid levels are often elevated in patients with leukemia and lymphomas, and they may be further raised by treatment with a variety of agents. This phenomenon is a well known clinical entity and occasional case reports have described the complications that may occur from it (7, 8, 10, 12).

In order to clarify the nature of this disturbance and to differentiate, if possible, the role of the primary disease from the role of therapy in its production, we have undertaken a systematic study of uric acid metabolism in patients with leukemia.

### Material and Methods

The subjects of these studies were patients with diagnoses of chronic myelocytic leukemia, chronic lymphatic leukemia, and acute leukemia. All the patients were hospitalized throughout the period of study. A constant purine intake was not attempted. In most instances, however, the dietary purine was low, and in many cases caloric intake was low. In addition to the usual hematologic parameters of evaluation, daily total urine uric acid determinations were made. Serum uric acid was determined two to three times weekly. The method employed for the determination of urine uric acid was a modification of the method of Archibald (3). Duplicate dilutions of urine were set up. One specimen was incubated with 0.01 ml. of liquid uricase (Worthington) for 90

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47 Smellie R M S Humphrey G F Kay E R M and Davidson J N *Biochem J* 60 177 (1955)
48 Stavitsky A B *Proc Soc Exptl Biol Med* 67 225 (1948)
49 Valentine W N Craddock C G Jr and Lawrence J S *Blood* 3 729 (1948)
50 Watson J D and Crick F H C *Nature* 171 737 (1953)

**Results and Discussion**

From the data available certain tentative hypotheses have been formulated about the mechanism of the uric acid abnormality in leukemia and the relationship of this mechanism to the therapeutic measures which have been found to have some favorable effect upon the disease

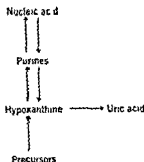



FIG 2 Simplified presentation of pathway of nucleic acid and uric acid synthesis

It has been conventionally assumed that in the effective therapy of leukemia lysis of large numbers of leukocytes and degradation of their nucleic acid purines into the end product uric acid are the means by which hyperuricemia and uricosuria are produced Although this mechanism is an important one under some circumstances there is reason to believe that there are other more complex metabolic pathways which are highly important in understanding this problem Figure 1 represents a simple but conventional concept of the normal route of nucleic acid synthesis incorporating into the same scheme the route of endogenous uric acid synthesis which partly parallels that of nucleic acid (5) Although it is thought that the bulk of uric acid in man comes from the breakdown of nucleoprotein several studies (1 2 9) have demonstrated *de novo* synthesis of uric acid in the human subject When $N^{15}$ labeled glycine is given there is prompt incorporation of a small portion of it into uric acid in a time interval which is too short to allow for incorporation into nucleic acid and subsequent degradation The magnitude of this prompt incorporation varies—it is notably increased in gout The evidence however seems clearly to indicate that the mechanism for *de novo* synthesis exists There is in this system production of a fairly constant amount of uric acid and presumably a fairly constant amount of nucleic acid subject to the requirements of growth and other situations requiring accelerated cellular proliferation Figure 2 is a simpler scheme of representing the same idea The sum

minutes at 37 to 45. Then both specimens were subjected to color development and the optical densities read at 660 m$\mu$ in a Coleman Junior spectrophotometer. The difference in optical densities between the uricase treated and the untreated specimens was considered to represent true uric acid. Serum uric acid determinations were performed in a similar manner, except that only occasional specimens were subjected to the incubation with uricase.

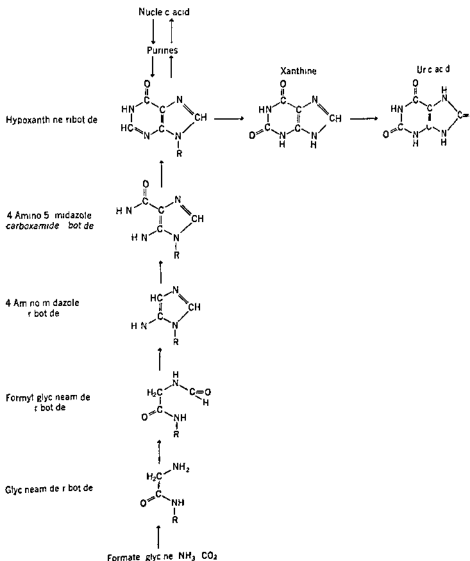



FIG. 1. Normal pathway of nucleic acid and uric acid synthesis.

myelocytic leukemia previously untreated During this period he was given only Fumigillin which had no effect on his disease Throughout this time his serum and urine uric acids were elevated above normal His white blood count was elevated but stable Similar observations have been made in other cases of chronic myelocytic leukemia It has not been possible to observe cases of acute leukemia with high leukocyte counts for such long periods of time with no treatment but briefer studies indicate that in that type of disease also the uric acid elevation is often though not always present prior to the institution of therapy It has not been possible to correlate differences in uric acid levels with differences in cellular type nor has it been possible to correlate the differences with subsequent response to therapy In untreated chronic lymphatic leukemia uric acid excretion has been found to be within normal limits

In studying the effects of therapy on uric acid our attention has been focused primarily on the antimetabolites and specifically on any differences that might be observed between different classes of antimetabolites Figure 4 identifies the site of action of three of these as demonstrated in bacterial and pigeon liver systems (4 6 11)

The purine analogs have been presumed to block the conversion of hypoxanthine to adenine the antifolics block the conversion of 4 amino 5 imidazole carboxamide to hypoxanthine The mustards and radiation have been considered to exert a direct effect on sensitive cells and to act by a destructive mechanism or by specifically inhibiting mitosis Thus this synthetic pathway is not blocked but the cells do not become available which might incorporate the purine being synthesized

If these observations are also valid for the situation which exists in the human subject with leukemia then qualitative and quantitative differences in uric acid excretion during therapy might be expected to occur Some of the studies which we have performed on human patients with leukemia have seemed to substantiate this hypothesis

Figure 5A represents the study of a patient with chronic myelocytic leukemia in whom as expected the uric acid excretion was elevated prior to therapy Treatment with 6 mercaptopurine produced a gradual fall in white blood count with a continued elevation of urine uric acid When the white count had fallen to normal levels with control of the disease the urine uric acid was normal

If we refer to the previously mentioned path of metabolism (Fig 5B) we may construe this as a block in synthesis of nucleic acid with continued unimpaired production of uric acid subsiding with control of the disease

plified scheme may serve as a basis for discussion of these mechanisms

In untreated leukemia there is a general acceleration of these synthetic pathways associated with increased formation of leukocytes containing

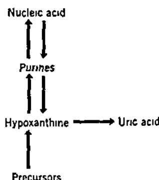



FIG 3A Pathway of nucleic acid and uric acid synthesis in untreated leukemia

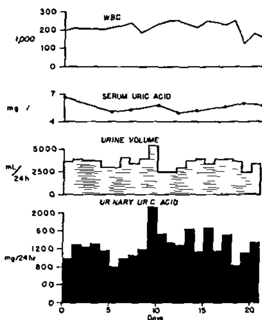



FIG 3B Study of patient with chronic myelocytic leukemia untreated

therefore increased amounts of nucleic acid Along with the generalized acceleration of this pathway there is increased production of uric acid and this is reflected in the elevated serum and urine uric acids seen in untreated leukemia This is shown diagrammatically in Fig 3A

Figure 3B is a graph illustrating this situation in a patient with chronic

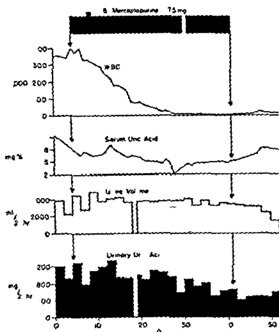



Fig 5A Study of patient with chronic myelocytic leukemia treated with 6-mercaptopurine

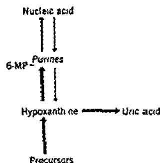



Fig 5B Pathway of nucleic acid and uric acid synthesis in leukemia treated with 6-mercaptopurine

Figure 6A represents the study of another patient with chronic myelocytic leukemia in whom urine uric acid was elevated prior to treatment. Amethopterin produced a rapid fall in white blood count accompanied by a rapid fall in urine uric acid. This may be interpreted as a block in the synthetic pathway at a point where both nucleic acid and uric acid synthesis are impaired (Fig. 6B).

Fig. 4. Site of action of antimetabolites as demonstrated in bacterial and pigeon liver systems.

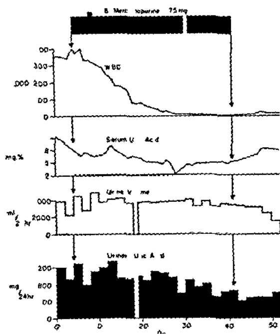



Fig 5A Study of patient with chronic myelocytic leukemia treated with 6 mercaptopurine

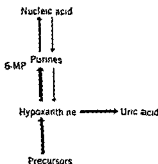



Fig 5B Pathway of nucleic acid and uric acid synthesis in leukemia treated with 6-mercaptopurine

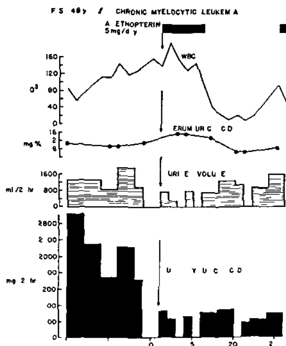



Fig 6A Study of patient with chronic myelocytic leukemia treated with amethopterin

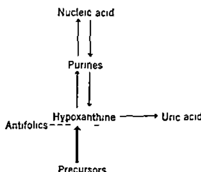



Fig 6B Pathway of nucleic acid and uric acid synthesis in leukemia treated with antifolics

Figure 7 is a sequential study of amethopterin and 6-mercaptopurine in a patient with chronic myelocytic leukemia Although there are quantitative differences between the degrees of activity of disease in the two studies as well as between the urine uric acids in the two studies it can be seen that there is also a qualitative difference in that the urine uric acid decreased while the white blood count was falling

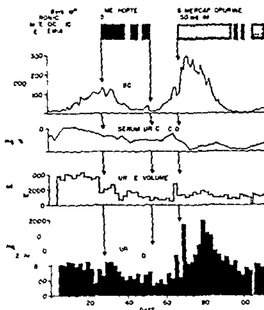



Fig 7 Study of patient with chronic myelocytic leukemia treated with amethopterin and 6-mercaptopurine

under the influence of amethopterin and rose while the count fell under the influence of 6-mercaptopurine

Figure 8A demonstrates a study of a patient with chronic myelocytic leukemia treated with amethopterin which produced a fall in urine uric acid and serum uric acid Subsequent x ray therapy to the spleen while the patient was still receiving amethopterin produced a second fall in white blood count along with a rise in urine and serum uric acid This would suggest that there is a fundamental difference in mechanism between these two types of treatment One might infer from these data that amethopterin can effectively block synthesis of uric acid but that this block is not applicable in respect to uric acid derived from degradation of nucleic acid (Fig 8B)

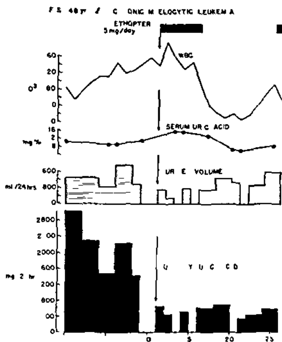



Fig 6A Study of patient with chronic myelocytic leukemia treated with amethopterin

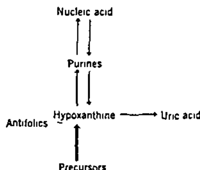



Fig 6B Pathway of nucleic acid and uric acid synthesis in leukemia treated with antifolics

2 to 3 weeks later This study tends to confirm the impression that the uric acid abnormality is related to the presence of active leukemia more closely than to change in the number of circulating leukocytes

Figure 10A illustrates the observations in a 44 year old man with acute leukemia treated with Meticorten It can be seen that his urine uric acid

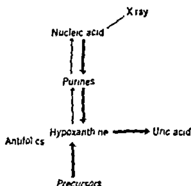



FIG 6B Pathway of nucleic acid and uric acid synthesis in leukemia treated with antifolics and x ray therapy

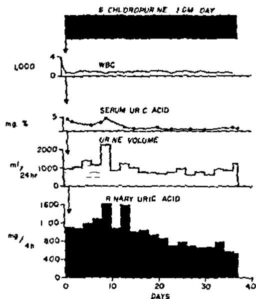



FIG 9 Study of patient with acute stem cell leukemia treated with 6-chloropurine

A further interesting factor in assessing the significance of uric acid in relation to the metabolic aspects of leukemia arises in the study of "aleukemic" leukemia Figure 9 represents a study of a 15 year old girl with acute stem cell leukemia During the early part of this study urine uric acid was elevated After several weeks of treatment with 6 chloropurine there was a fall in urine uric acid along with clinical and hematologic improvement The end of this study coincides with the patient's discharge from the hospital Complete hematologic remission occurred

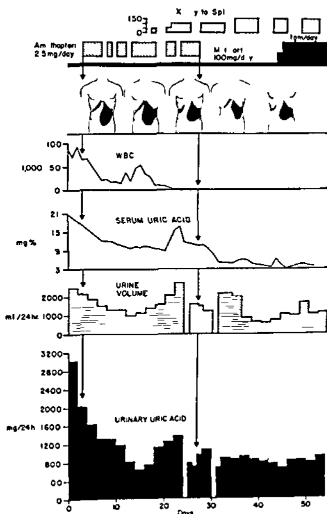



FIG 8A Study of patient with chronic myelocytic leukemia treated with amethopterin and x ray therapy

which was normal during the pretreatment period rose markedly with the administration of Meticorten while the leukocyte count was falling Subsequently in spite of the continued administration of meticorten the urine uric acid was within normal limits One may infer that the marked rise in urine uric acid during the period of fall in leukocyte count was due to the destructive effect of Meticorten on the cells with degradation of their nucleic acid Although the mechanism of action

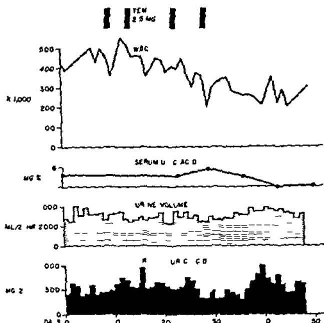



*Fig 11A Study of pati nt with chronic lymphatic leukemia treated with TEM*

of adrenal corticosteroids in producing remission in leukemia is not clear it seems that the mechanism by which they produce this type of abrupt fall in leukocytes is through lysis of cells similar to the lysis of lymphoid tissue which has been described after steroid administration In this respect they may be considered to be analogous to HN2 and x rays in the manner of which they produce a decrease in leukocytes and in their *effect on uric acid excretion (Fig 10B)* There are of course other effects of steroids on uric acid metabolism which are not however *germane to this discussion*

As was previously noted we have found uric acid excretion in chronic lymphatic leukemia to be normal or only slightly elevated Figure 11A

METICORTEN 1GM DAY

200
150
X 1,000 100
50
0
WBC

12
MG % 8
4
SERUM URIC ACID

7000
2000
1600
MG/24HR
1200
800
400
0
DAYS 0 5 10 15 20 25 30
URINARY URIC ACID

FIG 10A Study of patient with acute leukemia treated with Meticorten

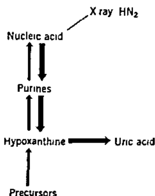



FIG 10B Pathway of nucleic acid and uric acid synthesis in leukemia treated with x ray therapy or HN2 (Adrenal corticosteroids should be included with HN2 and x rays in this diagram)

prolonged survival or reutilization of complex DNA fragments could explain the ineffectiveness of antimetabolites which are functioning at a simpler level On the other hand treatment with nitrogen mustard or x rays destroys cells and thus is not concerned with this mechanism After that type of treatment the uric acid rises

Although the studies presented form only a small part of the data available on this problem and only a minute part of the work which must still be done perhaps we may formulate some tentative hypotheses from these studies

1 It appears that there is a general increase in uric acid metabolism in leukemia which parallels and is part of a similar increase in nucleic

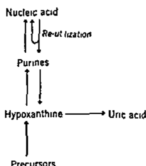



Fig 11C Pathway of nucleic acid and uric acid synthesis in chronic lymphatic leukemia showing reutilization of DNA

acid metabolism This does not occur however in chronic lymphatic leukemia

2 This increase is roughly proportional to activity of the disease and *is not necessarily correlated with the number of circulating leukocytes*

3 Leukemia in remission or "control is characterized by normal serum and urine uric acid

4 During the response to treatment the pattern of uric acid excretion *differs with different therapeutic agents Thus (a) the antifolics appear* to produce a fall in uric acid due to interruption of purine synthesis at *a relatively simple level (b) the purine analogs produce a rise in uric* acid due to interruption at a higher level at which the purine ring has already been formed and may be utilized for uric acid synthesis but not for nucleic acid synthesis (c) nitrogen mustard radiation and adrenal corticosteroids produce a rise in uric acid related not to interruption of synthesis but to destruction of cells and degradation of nucleic acids

is an example of such a patient It can be seen that in the period before treatment was given the urine uric acid was normal Subsequent treatment with TEM produced very little response in the disease and there was only a slight rise in urine uric acid Another patient (Fig 11B) in whom a fall in leukocyte count was achieved with TEM exhibited a definite rise in urine uric acid There is evidence that the survival time of the lymphocyte in chronic lymphatic leukemia is much longer than

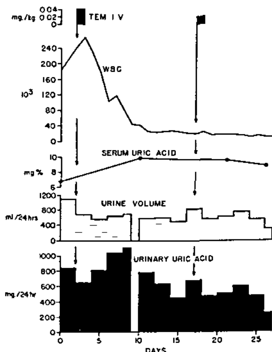



Fig 11B Study of patient with chronic lymphatic leukemia treated with TEM

that of the granulocyte Just how much longer is not known but recent work of Hamilton (personal communication) suggests that the lymphocyte survives for months or that it reutilizes large fragments of DNA without the necessity to synthesize DNA from simple precursors In either case the result would be a marked decrease in the turnover of simple precursors and nucleic acid degradation products Figure 11C incorporates this idea into the simple scheme we have been using It is interesting to consider the possible relationship of the low uric acid in chronic lymphatic leukemia to the slow turnover of DNA and its precursors One might also consider the relation of these two factors to the failure of the antimetabolites to be effective in this disease Either

# 24

# Nucleic Acids, Nucleases, and Nuclease Inhibitors in Leukemia

JOHN J WILL HELEN S GLAZER and RICHARD W VILTER [1]

Department of Internal Medicine College of Medicine
University of Cincinnati Cincinnati Ohio

The cells of acute and chronic leukemia have the capacity to divide repeatedly and to form new protein for their offspring Division is controlled by DNA containing chromosomes protein formation by the RNA containing cytoplasm and nucleoli Therefore we have investigated the nucleic acids their purine and pyrimidine bases and some of the enzyme systems responsible for their degradation and synthesis in the blood and bone marrow of normal persons and patients with leukemia and nonleukemic leukocytoses These studies are by no means complete but are being reported as a preview RNA has been determined by the orcinol method (1 13) DNA by the indole reaction (3) and the amounts have been expressed in terms of the average bone marrow cell The purine and pyrimidine bases have been quantitated by a chromatographic procedure adapted to bone marrow analyses by our laboratory staff but based on the methods of Vischer and Chargaff (17) and others No difference in cellular nuclease content of tumor cells compared with normal cells has been found by Greenstein (8) so this enzyme has not been investigated as yet

The cellular content of RNA is increased in acute leukemia Our initial results indicate that in this disease the average bone marrow cell contains about 0 5 $\times$ 10[6] mg as compared with 0 2 to 0 3 $\times$ 10[6] mg in the average bone marrow cells of normal individuals of persons with hyperplastic marrows and of persons with chronic lymphatic leukemia These results are similar to those found by other investigators who used

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[1] Report delivered by Dr Vilter

The clinical studies presented are shown as representative examples Other studies have been done with each of the agents discussed and in each case reveal results similar to those presented

Further studies are now under way to elucidate these mechanisms in the human and in nonmammalian species

## REFERENCES


1 Benedict J D Roche M Yu T F Bien E J Gutman A B and Stetten D Jr *Metabolism* 1 3 (1952)
2 Benedict J D Yu T F Bien E J Gutman A B and Stetten D Jr *J Clin Invest* 32 775 (1953)
3 Forsham P H Thorn G W Prunty F T G and Hills A G *J Clin Endocrinol* 8 15 (1948)
4 Greenberg G R *Federation Proc* 13 745 (1954)
5 Gutman A B *Ann Internal Med* 39 1062 (1953)
6 Karnofsky D A Graef I and Smith H W *Am J Pathol* 24 275 (1948)
7 Kravitz S C Diamond H D and Craver L F *J Am Med Assoc* 146 1595 (1951)
8 Merrill D *New Engl J Med* 222 94 (1940)
9 Muller A F and Bauer W *Proc Soc Exptl Biol Med* 82 47 (1953)
10 Richmond G H and Beardsley G D *Ann Internal Med* 39 1327 (1953)
11 Skipper H E *Ann N Y Acad Sci* 60 315 (1954)
12 Weisberger A S and Persky L *Am J Med Sci* 225 669 (1953)

# 24

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[1] Report delivered by Dr Vilter

the phosphorus method for their analyses (6 7 14 15) Since the methods used were entirely different the agreement seems to be quite remarkable Perhaps the RNA excess found in acute leukemic cells is the factor which makes possible continued protein synthesis

The amount of DNA is increased in acute leukemic cells also Pre liminary results from our laboratory indicate that the average bone marrow cells of patients with acute leukemia contain 0 8 $\times$ $10^8$ mg per cell as compared with values that range between 0 5 and 0 8 $\times$ $10^8$ mg per cell in persons with normal marrows hyperplastic marrows and marrows containing chronic lymphatic leukemia lymphocytes Com parable results have been obtained by other investigators who used the phosphorus method for their analyses (6 7 14 15) Any excess of DNA is probably the result of diploid and tetraploid chromosomes which are known to occur in leukemia and does not alter the concept that the resting haploid leukemia cells probably contain the same amount of DNA as the resting haploid normal cells

The pyrimidine bases are increased in acute leukemia also The average value for thymine in these cells is 0 7 $\times$ $10^8$ micromole per cell as compared with 0 4 to 0 6 $\times$ $10^8$ micromole per cell in normal mar rows hyperplastic marrows and marrows with chronic lymphatic leu kemia lymphocytes The average tentative values for uracil in acute leukemia are 0 16 $\times$ $10^8$ micromole per cell and 0 05 $\times$ $10^8$ micromole per cell in the other types of bone marrow studied The values for cytosine are 0 8 $\times$ $10^8$ micromole per cell in acute leukemia as com pared with 0 4 to 0 6 $\times$ $10^8$ micromole in the other types of marrow studied The purines are increased also The average for adenine in acute leukemia marrows is 0 8 $\times$ $10^8$ compared with 0 6 $\times$ $10^8$ micro mole in the other marrows studied The average for guanine in acute leukemia cells is slightly less than 0 6 $\times$ $10^8$ per cell as compared with slightly less than 0 5 $\times$ $10^8$ micromole in the cells of normal marrow hyperplastic marrow and chronic lymphatic leukemia marrow These initial results suggest that cytosine may be increased to a greater extent and guanine to a lesser degree than the others However these differ ences are not statistically valid as yet

In all these bone marrow analyses the ratio of purine to pyrimidine bases is very close to unity The ratio of thymine to DNA in acute leu kemia marrows is slightly higher than in the other conditions studied The ratio of uracil to RNA is the same in all There is a greater increase of RNA and uracil than of DNA and thymine in the cells of acute leu kemia in comparison with their normal relatives RNA/DNA ratios in acute leukemia are close to 0 6 compared with an average of 0 4 in a

normal individual Uracil/thymine ratios are 0 22 in acute leukemia compared with 0 18 in the other conditions studied These figures though provisional (since they are based on a study of 9 to 10 patients in each group) suggest that further analyses may indicate both a qualitative and a quantitative difference between the nucleic acids of the leukemic and the normal cell

DNAase an enzyme which depolymerizes the large DNA molecule (and perhaps polymerizes it) has not been measured by us but in other laboratories particularly Greenstein s it has been shown to exist in essentially the same amounts in animal leukemia and tumor cells as in normal cells However a DNAase inhibitor previously described by Henstell *et al* (9 10) has been investigated It has been quantitated by an alcohol precipitation test devised by McCarty (16) and by the methyl green test of Kurnick (11) in terms of residual crystalline pancreatic DNAase activity after the enzyme and the inhibitor have been incubated together What one measures is the amount of polymerized substrate DNA that finally is left after the enzyme and the inhibitor mixture have been allowed to act upon it This inhibitor is demonstrated most readily in normal cells There are smaller amounts in cells of chronic myeloid leukemia and even less in cells of chronic lymphatic and acute blastic leukemia The inhibitor concentrations can be expressed in arbitrary units called inhibitor units per 100 000 white cells From 4 to 25 inhibitor units is the normal range In chronic myeloid leukemia the inhibitor concentration is low averaging about 2 inhibitor units per 100 000 cells It is still lower in chronic lymphatic leukemia and in acute leukemia In the leukocytoses associated with infection in leukemoid reactions in the benign lymphocytoses of whooping cough and infectious mononucleosis and in myeloid metaplasia associated with myelofibrosis the inhibitor content is within the normal range These results are the same whether the test is performed by the methyl green method or by the alcohol precipitation technique

As patients with leukemia are brought under treatment and the peripheral blood picture improves the inhibitor concentration tends to return towards normal For instance in patients with chronic myeloid leukemia who are treated with myleran the inhibitor concentration rises as the total white blood cell count falls numbers of immature cells decrease and normal polymorphonuclear cells appear It may be important however that at a time when the number of cells is decreasing rapidly and they are becoming more mature the inhibitor content remains low The rise occurs considerably later This type of observation has been repeated many times On occasion a relapse in the leu

kemic process may be predicted several weeks or more in advance by a fall in the inhibitor concentration of the cells

In chronic lymphatic leukemia a change in the inhibitor concentration in the cells has not been demonstrated as the white count falls and patients go into partial remission Usually as the total white cell count is reduced the percentage of lymphocytes remains elevated and the inhibitor concentration remains low Apparently in this circumstance a new population of more normal lymphocytes does not appear in the peripheral blood and therefore the inhibitor concentration does not rise

If one selects patients with equally high total white blood cell counts and lymphocyte percentages due to chronic lymphatic leukemia whooping cough and infectious mononucleosis and if one determines the inhibitor concentration in each of these lymphocyte populations it becomes apparent that there is a great deal of difference between them Patients with chronic lymphatic leukemia will have cells with an extremely low inhibitor content whereas patients with infectious mononucleosis and whooping cough will have cells with normal inhibitor content This indicates a fundamental difference in the two types of lymphocytes though their appearance may be very similar

This inhibitor of DNAase is a very labile substance It disappears after 24 to 48 hours at –4 and after 15 minutes at 56 Its effect is instantaneous and reversible It acts over a pH range from 3 to 11 It is nondialyzable is destroyed by trypsin and is precipitated with the ribo nucleic acid fraction It can be adsorbed on $BaSO_4$ and eluted with Na citrate An atmosphere of nitrogen does not protect it This substance has not yet been fully characterized although it probably contains protein it is not an enzyme but inactivates DNAase without destroying it

Other inhibitors of DNAase have been described Kurnick (12) has investigated a protein inhibitor which is apparently involved in the lupus cell phenomenon It is in the cytoplasm but in very much smaller concentrations It is quite stable and is species specific Unlike the inhibitor we have investigated it is found in larger amounts in leukemia cells and smaller amounts in normal cells Zamenhof and Chargaff (18) described a species specific DNAase inhibitor obtained from yeast which was protein in nature and quite unstable It did not attack pancreatic DNAase Dabrowska *et al* (4 5) described an unstable protein inhibitor of DNAase obtained from avian and mammalian organs It too lacked species specificity Bernheimer and Ruffier (2) reported on an inhibitor obtained from the streptococcus It differed from the pre

ceding substances in that it was destroyed by ribonuclease but not by trypsin Only the inhibitors of Henstell and of Kurnick have been studied in leukemia, and it is apparent that these are entirely different substances

They may however play an important role in the metabolism of the leukemia cell the one we have studied might be responsible for the unlimited capacity for division demonstrated by the cells of acute leukemia victims since in these cells DNAase is relatively uninhibited This situation may be partially responsible for the recurring degradation and synthesis of polymerized DNA

Summary

Cells of acute leukemia contain more DNA and RNA than normal bone marrow cells RNA excess is greater than DNA excess so the RNA DNA ratio is high All the purines and pyrimidines are increased also cytosine to a greater extent and guanine to a lesser extent than the others These data suggest that the requirements for rapid cell division and protein formation are met in this way

The DNAase content of tumor and leukemia cells is the same as in normal cells but a DNAase inhibitor is deficient in the cells of chronic lymphatic leukemia and acute leukemia Cells of chronic myeloid leukemia have a somewhat higher concentration which, however does not equal the amounts found in normal cells cells from patients with lymphocytic or granulocytic leukemoid reactions leukocytoses or myeloid metaplasia This inhibitor is a very labile constituent of the cytoplasm and may be implicated in chemical reactions that impart to the leukemic cells the potentiality for unlimited growth and reproduction

REFERENCES

1 Albaum H G and Umbreit, W W *J Biol Chem* 167 369 (1947)
2 Bernheimer A W and Ruffer N K *J Exptl Med* 93 399 (1951)
3 Cerotti C *J Biol Chem* 198 297 (1952)
4 Cooper E J Trautmann M L and Laskowski M *Proc Soc Exptl Biol Med* 73 219 (1950)
5 Dabrowska W Cooper E J and Laskowski M *J Biol Chem* 177 991 (1949)
6 Davidson J N Leslie I and White J C *Lancet* 1 1287 (1951)
7 Davidson, J N Leslie I and White J C *J Pathol Bacteriol* 63 471 (1951)
8 Greenstein, J P *in* Biochemistry of Cancer 2nd ed Academic Press New York, 1954
9 Henstell H H and Freedman R I *Cancer Research* 12 341 (1952)
10 Henstell H H Freedman R I and Ginsburg, B *Cancer Research* 12 346 (1952)

11 Kurnick N B *Arch Biochem* 29 41 (1950)
12 Kurnick N B Schwartz L I Pariser S and Lee S L *J Clin Invest* 32 193 (1953)
13 Mejbaum W *Z physiol Chem* 258 117 (1939)
14 Menten M L and Willms M *Cancer Research* 13 733 (1953)
15 Menten M L Willms M and Wright W D *Cancer Research* 13 729 (1953)
16 McCarty M *J Exptl Med* 90 543 (1949)
17 Vischer E and Chargaff E *J Biol Chem* 176 703 (1948)
18 Zamenhof S and Chargaff E *J Biol Chem* 180 727 (1949)

# 25

## Studies on Cysteine Metabolism in Leukemia

AUSTIN S. WEISBERGER
University Hospitals, Cleveland, Ohio

This report will deal with some aspects of sulfhydryl metabolism as they may pertain to leukemia with special reference to the apparently unique role of the sulfhydryl amino acid L-cysteine. An attempt will be made to summarize some of the evidence for the importance of sulfhydryl compounds in general cellular metabolism as well as in disorders of leukocytes. Some relationships of L-cysteine metabolism to leukopoiesis and leukemia as well as the effects of an analog of cysteine on leukemia will also be presented. Throughout the discussion the terms cysteine and cystine will frequently be used interchangeably since they are readily interconverted and may be considered as a single amino acid in metabolism.

### General Aspects of Sulfhydryl Compounds in Cell Growth and Division

The importance of sulfhydryl compounds in cellular metabolism and in cell growth and division in general has been repeatedly emphasized. Sulfhydryl compounds in the reduced state (SH) have been found to stimulate mitosis in a wide variety of cells and tissues. Substances that oxidize sulfhydryl compounds (SH → S S) or combine with SH groups to inactivate them have been found to inhibit cell division. The literature pertaining to this subject has been reviewed extensively by Brachet (8), by Contopoulos and Anderson (10), and by Barron (2, 4).

The mechanisms by which sulfhydryl compounds may affect cell metabolism and cell growth and division are not known. Sulfhydryl compounds are universally distributed in cells and may thereby regulate cellular oxidation-reduction systems (5, 17, 19, 25). Sulfhydryl compounds are also known to be essential for the activity of many enzymes (2, 3, 6, 8). Brachet (8) has postulated that some of these enzymes may be involved in the synthesis of ribose and deoxyribose nucleic acids

Vitamin $B_1$ which has an important role in cellular metabolism may function in forming SH compounds from their disulfide (S S) precursors and thereby act directly or indirectly in the activation of SH enzymes (12 13) Ling and Chow have shown that $B_1$ deficiency in rats results in a diminution of the sulfhydryl content of blood They suggest that $B_1$ may activate SH enzymes and that these enzymes may increase the utilization of carbohydrates thereby providing energy and building materials for the synthesis of cellular constituents (22)

Sulfhydryl compounds have been implicated in other fundamental synthetic processes such as the transfer of labile methyl groups from methionine (9) Sulfhydryl amino acids may also participate in protein synthesis by forming intermediate compounds during biosynthetic processes (15 39) Barron (5) has suggested that sulfhydryl compounds may be present in cells as soluble compounds which affect cellular respiration and that they may also affect cellular function by being present as fixed groups in enzyme protein complexes

Thus sulfhydryl compounds may be involved in cell growth by furnishing *energy for metabolism by activating essential SH enzymes* or by forming intermediates during biosynthetic processes

### The Sulfhydryl Content of Normal and Leukemic Leukocytes

Sulfhydryl compounds have been implicated in the growth of malignant tumors in general (14 26 28 30 40) Thus tumor tissues have been found to contain a higher sulfhydryl concentration than normal tissues whereas a decrease in the amount of available sulfhydryl may result in the inhibition of tumor growth

*Similarly abnormalities of sulfhydryl metabolism have frequently* been implicated in disorders of leukocytes The sulfhydryl content of normal and leukemic leukocytes has been studied by several investigators Glutathione (GSH) appears to be the most important sulfhydryl compound present intracellularly and most studies have been on the content of GSH in leukocytes The values obtained in different studies cannot be directly compared because of differences in technique and standards of expression However many types of leukemic leukocytes have been reported to contain a higher concentration of GSH than normal leukocytes (7 10 24 31) Although most investigators have found a significant elevation in chronic myeloid leukemia there is no unanimity about the findings in chronic lymphatic leukemia and in acute leukemia Both high and low concentrations of GSH have been reported in the leukocytes of chronic lymphatic leukemia and in acute leukemia Contopoulos and Anderson (10) found the highest GSH concentration in

the most immature leukemic leukocytes and suggest that the GSH concentration may be a criterion of the malignancy of the leukemic process

The elevations of GSH reported in leukemia appear to be independent of the height of the leukocyte count Parker and Kracke (23) found no elevation of GSH in physiologic leukocytosis which supports the suggestion that increased GSH concentration may be correlated with degree of immaturity Low GSH levels have been found in leukocytes in leukopenic states and in aplastic anemia (10 21 23) These values are also independent of the cell count

It has frequently been suggested that the elevated GSH concentration in immature leukocytes may act as a growth stimulus Hueper (18) and Kandel and LeRoy (20) believe that such an assumption is unwarranted The latter were unable to correlate changes in GSH concentration with any variations in leukocytes Similarly Hardin *et al* (16) were unable to corroborate the findings of elevated GSH concentrations in leukemic leukocytes reported by others

It has been observed however that substances such as irradiation nitrogen mustard arsenic gold and benzol which inactivate sulfhydryl groups tend to produce a leukopenia Furthermore diets low in sulfhydryl amino acids result in a reduced incidence of induced leukemia in animals (41) Diets low in cysteine or in methionine (11) which is a cysteine precursor result in leukopenia in animals The leukopenia associated with either $B_1$ deficiency or folic deficiency may be associated with alterations in the effect of these substances on sulfhydryl metabolism (12 13 22 27) Thus although there is no unanimity of opinion there is a great deal of evidence supporting the suggestion that sulfhydryl compounds may have an important role in normal and abnormal leukopoiesis

## The Importance of L Cysteine in the Metabolism of Leukocytes

The sulfhydryl amino acid L cysteine appears to have an especially important role in the metabolism of leukocytes Thus L cysteine modifies the severe leukopenia characteristically induced by nitrogen mustard (HN2) No such protective effect occurs if L cysteine is administered after the injection of HN2 (Table I) (32) Certain structural components appear to be essential for this protective effect (33 35) Thus many highly reactive compounds with structures closely related to L cysteine fail to modify the leukopenia induced by HN2 and only compounds containing a sulfhydryl amino and carboxyl group in close apposition are effective (Fig 1)

Vitamin $B_1$ which has an important role in cellular metabolism may function in forming SH compounds from their disulfide (S S) precursors and thereby act directly or indirectly in the activation of SH enzymes (12 13) Ling and Chow have shown that $B_1$ deficiency in rats results in a diminution of the sulfhydryl content of blood They suggest that $B_1$ may activate SH enzymes and that these enzymes may increase the utilization of carbohydrates thereby providing energy and building materials for the synthesis of cellular constituents (22)

Sulfhydryl compounds have been implicated in other fundamental synthetic processes such as the transfer of labile methyl groups from methionine (9) Sulfhydryl amino acids may also participate in protein synthesis by forming intermediate compounds during biosynthetic processes (15 39) Barron (5) has suggested that sulfhydryl compounds may be present in cells as soluble compounds which affect cellular respiration and that they may also affect cellular function by being present as fixed groups in enzyme protein complexes

Thus sulfhydryl compounds may be involved in cell growth by furnishing energy for metabolism by activating essential SH enzymes or by forming intermediates during biosynthetic processes

## The Sulfhydryl Content of Normal and Leukemic Leukocytes

Sulfhydryl compounds have been implicated in the growth of malignant tumors in general (14 26 28-30 40) Thus tumor tissues have been found to contain a higher sulfhydryl concentration than normal tissues whereas a decrease in the amount of available sulfhydryl may result in the inhibition of tumor growth

Similarly abnormalities of sulfhydryl metabolism have frequently been implicated in disorders of leukocytes The sulfhydryl content of normal and leukemic leukocytes has been studied by several investigators Glutathione (GSH) appears to be the most important sulfhydryl compound present intracellularly and most studies have been on the content of GSH in leukocytes The values obtained in different studies cannot be directly compared because of differences in technique and standards of expression However many types of leukemic leukocytes have been reported to contain a higher concentration of GSH than normal leukocytes (7 10 24 31) Although most investigators have found a significant elevation in chronic myeloid leukemia there is no unanimity about the findings in chronic lymphatic leukemia and in acute leukemia Both high and low concentrations of GSH have been reported in the leukocytes of chronic lymphatic leukemia and in acute leukemia Contopoulos and Anderson (10) found the highest GSH concentration in

been completed he has observed that many other sulfhydryl compounds are ineffective

The effects of L cysteine depletion are also suggestive of the importance of this amino acid in the metabolism of leukocytes As previously noted animals fed on diets deficient in cysteine or its metabolic precursor methionine develop leukopenia (11) Lack of cystine in the diet reduces the incidence of leukemia in animals whereas addition of cystine increases the incidence (41) This is not a nonspecific effect due to dietary restriction with subsequent weight loss since similar restrictions of lysine and tryptophan have no effect on the incidence of leukemia It has also been observed that cultures of human bone marrow on synthetic media deficient in either L cysteine or L cystine exhibit rapid degeneration of the leukocytes Addition of either L cysteine or L cystine to the media results in a protective effect especially of granulocytic leukocytes (1)

Studies with sulfur 35 ($S^{35}$) labeled L cystine demonstrate that the leukocytes of acute leukemia and of chronic myeloid leukemia exhibit a much more rapid incorporation of L cystine than normal leukocytes (34) Tracer amounts of $S^{35}$ L cystine were administered orally to normal individuals and to patients with acute leukemia and the amount of radioactivity present in the leukocytes was determined at intervals More radioactivity appeared in the leukocytes of acute leukemia within 20 minutes than was present in normal leukocytes in 48 hours (Fig 2) A similar rapid incorporation into leukocytes was observed in chronic myeloid leukemia but not in chronic lymphatic leukemia Incorporation of $S^{3}$ L cystine also occurs when leukemic leukocytes are incubated with the radioactive amino acid *in vitro* Radioactivity is present in several fractions in both acid soluble and acid insoluble extracts of these leukemic leukocytes (Fig 3)

This rapid turnover of an amino acid by immature leukocytes is not limited to L-cystine and is probably a manifestation of the rapid metabolic turnover characteristic of immature cells in general In view of the other observations on the importance of L cysteine and L cystine in leukopoiesis however the avidity of immature leukocytes for L cystine may have greater significance

## The Effects of an Analog of Cystine on Leukemia

The preceding observations suggest that a decreased availability of sulfur in the form of L cysteine or of L cystine may have important effects on leukocytes Since leukemic leukocytes have a much more rapid turnover of L cystine than do normal leukocytes they may be more sus

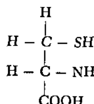



Fig 1 Structure of L cysteine Any modification of the reactive sulfhydryl amino or carboxyl group results in a loss of protective effect against mustard induced leukopenia

Compounds in which there is alteration or substitution of any of these reactive groups have no demonstrable effect in preventing HN2 induced leukopenia Removal of any of these reactive groups from the molecule also results in loss of a protective effect Neither the reducing action of a compound nor the presence by themselves of either free SH amino or carboxyl groups can be correlated with the ability of a compound to prevent leukopenia when injected prior to HN2 Thus of the many compounds studied only L cysteine and to a lesser extent homocysteine and glutathione exert any protective influence

The specificity of cysteine protection is best illustrated by experiments with the optical isomers of cysteine (Table I) When HN2 is injected

TABLE I
Effect of Optical Isomers of Cysteine on Neutropenia Induced by Nitrogen Mustard (HN2)

| | Maximum decrease in neutrophils % of initial value |
|---|---|
| HN2 | 83 |
| L Cysteine prior to HN2 | 31 |
| D Cysteine prior to HN2 | 82 |
| L Cysteine after HN2 | 82 |

into rabbits in a dose of 2 5 mg/kg body weight the absolute neutrophil count decreases 83% of the initial value in 4 days If 500 mg of L cysteine per kilogram body weight is administered prior to the HN2 the decrease in neutrophils is only 31% of the initial value If the same amount of D cysteine is administered prior to the HN2 no protective effect is obtained and the neutrophil count decreases 82% of the initial value

Moeschlin (personal communication) has observed that L cysteine also protects against the leukopenia induced by benzol in animals Although studies on the structural specificity required for this protection have not

ceptible to depletion of cystine than normal leukocytes Accordingly the effect of an analog of cystine on leukemia was studied

Potential blocking analogs were selected by determining the ability of various compounds to decrease the influx of $S^{35}$ L-cystine into leukemic leukocytes *in vitro* (38) Leukemic leukocytes were exposed to a num

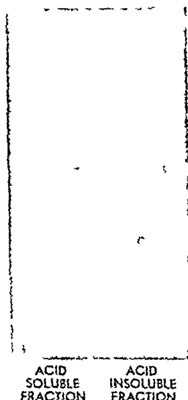



Fig 3 Radioautographs of chromatograms obtained from trichloroacetic acid fractions of pooled leukocytes of acute monocytic leukemia after incubation with radioactive L-cystine

ber of compounds with structures closely related to either cysteine or cystine prior to incubation with radioactive L cystine It was found that only a few such compounds were capable of decreasing the influx of radioactive L cystine into the leukemic leukocytes Many compounds closely related to cysteine were ineffective in decreasing the incorpora tion of $S^{35}$ L cystine Even alteration of the position of the SH group within the molecule renders it ineffective (Fig 4) The specificity of

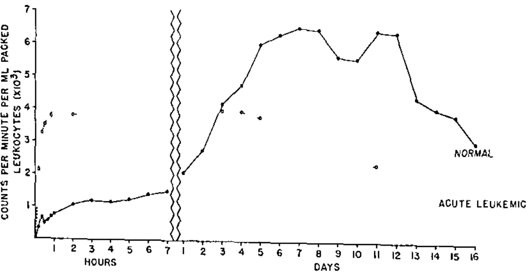



Fig 2 Comparative incorporation of $S^{35}$ L cystine by normal and acute leukemic leukocytes More radioactivity is present in the leukocytes of acute leukemia within 20 minutes than is present in normal leukocytes within 48 hours [Reproduced with the permission of the publishers from *Blood* 9 1082 (1954) ]

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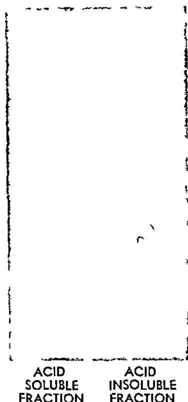



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this inhibitory effect is best illustrated by the fact that preincubation with unlabeled L cysteine decreases the influx of $S^{35}$ L cysteine whereas preincubation with either D cysteine homocysteine or isocysteine is ineffective This suggests that highly specific intracellular receptors are involved

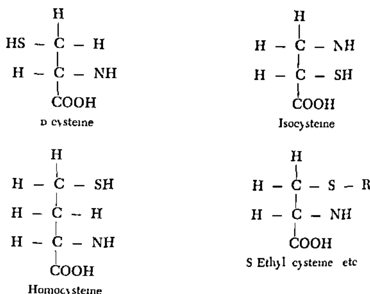



FIG 4 These compounds are ineffective in decreasing the incorporation of radioactive L cystine into leukemic leukocytes despite their close similarity to L cysteine

Of the compounds studied the most effective inhibitor was selenium cystine (diseleno dialanine) This compound has a structure identical with cystine except that selenium replaces the sulfur in the molecule (Fig 5) It is effective in low concentrations in decreasing the incorporation of radioactive cystine into leukemic leukocytes *in vitro* This inhibitory effect is not due solely to the presence of selenium since other selenium compounds are ineffective (38) In addition to its effects *in vitro* selenium cystine was also found to inhibit tumor growth in the intact animal (36)

The effect of this analog has been studied in eight patients with leukemia most of whom were in the terminal phases of their disease and were no longer responsive to the usual chemotherapeutic agents (Table II) (37) Two patients with acute leukemia two patients with chronic myeloid leukemia and one patient in the terminal blastic state of chronic myeloid leukemia responded with a significant fall in leukocyte count and decrease in spleen size One patient with blastic chronic mye

TABLE II

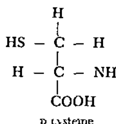
RESULTS OBTAINED WITH ORAL ADMINISTRATION OF SELENIUM CYSTINE IN PATIENTS WITH LEUKEMIA

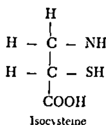
| Patient | Diagnosis | Decrease in WBC | Decrease in spleen size | Maximum WBC count cells/mm³ * | Lowest WBC count † cell /mm³ | Duration of therapy |
|---|---|---|---|---|---|---|
| 1 | Acute leukemia (stem cell) | + | + | 505 000 | 24 000 | 57 days |
| 2 | Acute leukemia (childhood) | + | + | 110 000 | 13 500 | 21 days |
| 3 | Chronic myeloid leukemia | + | + | 147 000 | 44 000 | 21 days |
| 4 | Chronic myeloid leukemia | + | + | 2_2 000 | 121,250 | 10 days |
| 5 | Blastic chronic myeloid leukemia | + | + | 113 750 | 17 100 | 14 days |
| 6 | Blastic chronic myeloid leukemia | 0 | 0 | _65 500 | 219 000 | 6 days |
| 7 | Acute leukemia (childhood) | 0 | 0 | 161 250 | 78 000 | 7 days |
| 8 | Chronic monocytic leukemia | 0 | 0 | 110 000 | 123 000 | 12 days |

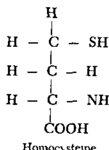
* Immediately prior to selenium cystine therapy

† During therapy with selenium cystine

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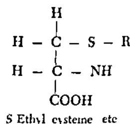




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chemotherapeutic agents and obtained only a transient drop in leukocyte count. Because of the continuing rise in leukocyte count and progressive increase in the size of the spleen and lymph nodes he was given selenium cystine orally in a dose of 100 mg daily The leukocyte count fell from 110 000 cells/mm³ to 13,500 cells/mm³ and the size of the spleen and lymph nodes decreased Selenium cystine was then discontinued because of toxic manifestations after which the leukocyte count again began to rise and the spleen and lymph nodes increased in size Subsequently he was again treated with 6-mercaptopurine and although he had previously failed to respond to this drug in the same dosage he obtained a very satisfactory remission

The response obtained with selenium cystine is not due solely to the presence of selenium in the molecule This is illustrated by the effect of another selenium compound on a patient with acute leukemia (Fig 7) The patient was a 39 year-old woman with acute stem cell leukemia During a 7-day period of observation the leukocyte count rose spontaneously from 214,250 cells mm³ to 390 000 cells/mm³ On selenium cystine (100 mg per day orally) the leukocyte count gradually declined and the spleen decreased in size The administration of selenium cystine was discontinued and she was then given an equivalent amount of diphenyl diselenide a selenium compound which is unrelated to cystine While receiving this selenium compound the patient s leukocyte count rose rapidly to over 500 000 cells/mm³ When selenium cystine therapy was again instituted the leukocyte count again fell sharply

The toxic manifestations associated with the administration of selenium cystine include severe nausea vomiting, anorexia glossitis and diarrhea Moderate drowsiness may occur Some of the selenium is excreted through the lungs and has a disagreeable pungent, garlic like odor These side effects make it an extremely unpleasant and difficult drug to administer No hepatic or renal damage was encountered either by ordinary function tests or at post mortem examination even in one patient who received over 5 g of selenium cystine in a 2 month period No irreversible toxicity was encountered in any of the patients treated Severe alopecia occurred in all patients and damage to the fingernails and toenails developed in three patients Normal hair and fingernail growth resumed when selenium cystine was discontinued The changes in hair and fingernails are particularly intriguing because of the known high cystine content of these appendages as well as the rapid cellular proliferation in nail beds and hair follicles The toxic manifestations of selenium cystine are very similar to those of aminopterin and it is possible that both of these compounds may have similar sites of action

loid leukemia one patient with acute leukemia and one patient with chronic monocytic leukemia failed to exhibit significant reduction in either the leukocyte count or spleen size Duration of therapy may be a factor in lack of response Thus an incomplete response (patient 4) or failure to respond (patients 6 7 and 8) occurred when therapy was carried out for less than a 2-week period Further data are needed to corroborate this observation

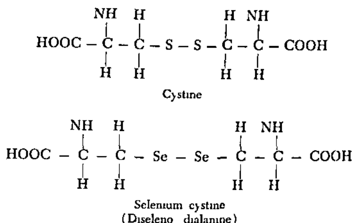



FIG 5 Selenium cystine has the same structural configuration as cystine except that selenium replaces the sulfur in the molecule

Although our experience with selenium cystine has been limited to these patients several remarkable effects have become evident Thus in patients responding to the analog the fall in leukocyte count once it occurs may be very rapid This was most apparent in acute leukemia where the total leukocyte count decreased as much as 75 000 cells/mm $^3$ in 24 hours In most of the patients the maximum rate of fall was approximately 100 000 cells/mm $^3$ in a 7 day period Immature leukocytes appear to be more sensitive than mature leukocytes to selenium cystine Thus in chronic myeloid leukemia myeloblasts and myelocytes disappear more rapidly than more mature leukocytes

Patients refractory to other chemotherapeutic agents may respond to selenium cystine One patient with acute leukemia (patient 2) who had become refractory to 6 mercaptopurine appeared to reacquire sensitivity to the purine analog after receiving a course of selenium cystine (Fig 6) The patient was a 13 year old boy who had been maintained in good remission for a period of 12 months on successive courses of cortisone aminopterin and 6 mercaptopurine At the end of this period of time he relapsed He was again treated with large amounts of these

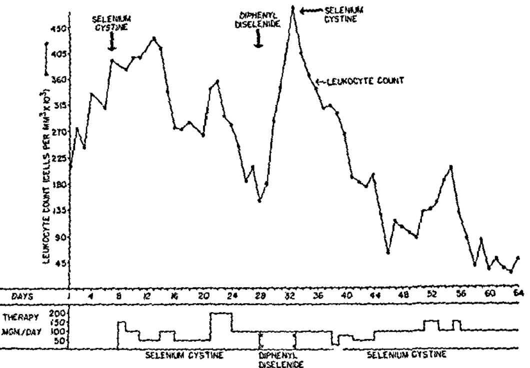



FIG 7 The effect of selenium cystine on acute stem cell leukemia in an adult During the initial period of observation the leukocyte count rose from 214 000 to 330 000 cells/mm³ On selenium cystine therapy the count then fell to 140 000 cells/mm³ When another selenium preparation diphenyl diselenide was substituted for the selenium cystine the count rose rapidly to 505 000 cells/mm³ When selenium cystine therapy was reinstituted the leukocyte count fell rapidly to 45 000 cells/mm³ Note the rapid rate of fall associated with the administration of selenium cystine (Reproduced with the permission of the publisher from *Blood* 11 19 (1956) )

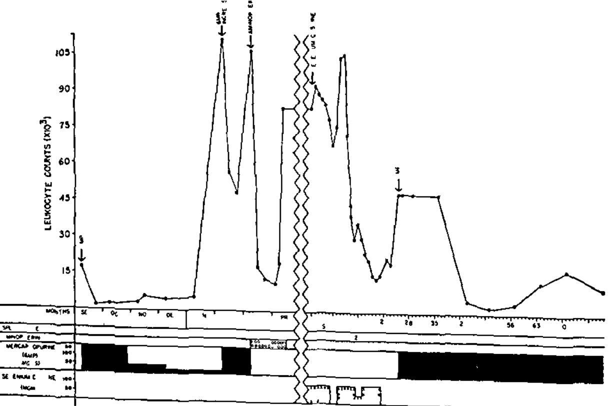



FIG 6 The effect of selenium cystine on a patient with acute leukemia The patient had been maintained with successive courses of cortisone aminopterin and 6-mercaptopurine for 12 months He then became re sistant to these chemotherapeutic agents but responded to sel ni m cystine w th a fall in leukocyte count and decrease in the size of lymph nodes and spleen Sub equ ntly l e reacqu d sen t v ty to 6 me aptopu ine and achi v d a satisfactory ren is on

## REFERENCES


1. Baldini M and Sacchetti C *Rev hematol* 8 3 (1953)
2. Barron E S G in "The Chemistry and Physiology of Growth" (A K Parpart ed) Princeton 1949
3. Barron, E S G *Advances in Enzymol* 11 201 (1951)
4. Barron E S G *Texas Repts Biol and Med* 11 653 (1953)
5. Barron E S G Nelson L and Ardao M I *J Gen Physiol* 32 179 (1948-1949)
6. Barron E S G and Singer T P *Science* 97 356 (1943)
7. Bichel J *Acta Med Scand* 124 160 (1946)
8. Brachet J "Chemical Embryology" Interscience Publishers New York 1950
9. Cantoni G *J Biol Chem* 189 745 (1951)
10. Contopoulos A N and Anderson H H *J Lab Clin Med* 36 929 (1950)
11. Dinning J S Payne L D and Day P L *Arch Biochem* 27 467 (1950)
12. Dubnoff J W *Arch Biochem* 27 466 (1950)
13. Dubnoff J W Federation Proc 9 168 (1950)
14. Hammett F S *Arch Pathol* 8 575 (1929)
15. Hanes C S Hird F J R and Isherwood F A *Nature* 166 288 (1950)
16. Hardin E B Valentine W N and Follette J H *Am J Med Sci* 228 73 (1954)
17. Hopkins F G and Morgan E J *Biochem J* 32 611 (1938)
18. Hueper W C *Arch Pathol* 17 218 (1934)
19. Kahn M and Goodridge F G "The Biological Significance of the Sulfhydryl Group" Chapter 23 Lea & Febiger Philadelphia 1928
20. Kandel E V and LeRoy G V *J Lab Clin Med* 24 669 (1939)
21. Kugell V C Koets P Schaffarzick, R W and Naugler W E *Stanford Med Bull* 13 284 (1955)
22. Ling C T and Chow B F *J Biol Chem* 202 445 (1953)
23. Parker F P and Kracke R R *Am J Clin Pathol* 6 41 (1936)
24. Platt R *Brit J Exptl Pathol* 12 139 (1931)
25. Rapkine L *Biochem J* 32 1729 (1938)
26. Shacter B Entenman C and Shimkin M B *J Natl Cancer Inst* 13 647 (1952)
27. Stekol, J A Weiss K W and Weiss S *Federation Proc* 10 252 (1951)
28. Stern K and Willheim R "The Biochemistry of Malignant Tumors" Reference Press Brooklyn 1943
29. Voegtlin C Johnson J M and Thompson J W *Public Health Repts (U S)* 51 1689 (1936)
30. Voegtlin C and Thompson J W *J Biol Chem* 70 801 (1926)
31. Weil E Aschkenasy A and Capron L *Le Sang* 13 705 (1939)
32. Weisberger A S and Heinle R W *J Lab Clin Med* 36 872 (1950)
33. Weisberger A S Heinle R W and Levine B *J Clin Invest* 31 217 (1952)
34. Weisberger A S and Levine B *Blood* 9 1082 (1954)
35. Weisberger A S and Storaasli J P *J Lab Clin Med* 43 246 (1954)
36. Weisberger A S and Suhrland L G *Blood* 11 11 (1956)
37. Weisberger A S and Suhrland L G *Blood* 11 19 (1956)
38. Weisberger A S Suhrland L G and Seifter J *Blood* 11 1 (1956)

It is apparent that small amounts of selenium cystine may have a rapid and striking effect on leukemic leukocytes The effectiveness of this selenium compound appears to depend to a great extent on its structural relationship to cysteine and is not due solely to the presence of selenium within the molecule The mechanism by which selenium cystine produces these profound effects is not known It may interfere with biosynthetic mechanisms of leukocytes (1) by competitively combining with intracellular receptors for cystine (2) by inactivating enzymes essential to the metabolism of leukocytes or (3) by serving as a vehicle by virtue of its structural identity with cystine permitting selenium to enter the cell where it exerts a toxic effect

The value if any of selenium cystine as a practical therapeutic agent in leukemia remains to be determined We have been unable to administer sufficient selenium cystine for a long enough period of time to determine whether or not more lasting or more complete remissions might be obtained This has been due in part to the difficulty in synthesizing the compound and in part to the severe nausea and vomiting associated with its administration It is hoped that other analogs of cystine may be found which are less toxic or that perhaps smaller courses of selenium cystine may be effective if used in combination with other chemotherapeutic agents

Although the clinical application remains to be determined these rather striking results of a blocking analog of cystine tend to confirm the importance of this amino acid in the metabolism of leukocytes

## Summary and Conclusions

There is considerable evidence that abnormalities of sulfhydryl metabolism may be implicated in disorders of leukocytes The sulfhydryl amino acid L cysteine appears to have an especially important role in the metabolism of leukocytes These observations led to a study of the effects of a decreased availability of cysteine or cystine on leukemia Selenium cystine which is an analog of cystine in which selenium replaces sulfur in the molecule was found to decrease the influx of radioactive cystine into leukemia leukocytes Furthermore selenium cystine produces a rapid fall in the leukocyte count and a decrease in spleen size in some patients with leukemia Although clinical application if any remains to be determined the results observed with this analog of cystine tend to confirm the importance of this amino acid in the metabolism of leukocytes

We have found that this inhibitor is particularly abundant in lymphocytes both mature and immature We would suggest that the findings that Dr Krakoff and Dr Hamilton reported on apparent reutilization of *nucleic acid fragments from DNA* may depend on the fact that lymphocytes are so abundant in this inhibitor This might prevent the complete breakdown of the nucleic acid before it has an op*portunity to be reutilized, as may occur in the case of the granulocytes*

DR HENRY S KAPLAN (San Francisco California) I should like to comment on part of Dr Vilter s presentation.

We have previously reported a similar observation of an increase in RNA content per cell and in RNA to DNA ratio in the lymphocytes of the thymus of irradiated mice during the course of lymphoid tumor induction. This of course is an interest*ing finding both in his material and in ours*

I think it is important to be aware of the pitfalls in the interpretation of such a finding It has not been very long since we graduated from the days of expressing results with regard to both DNA and RNA in terms of concentration in tissues I think most of us are now aware that such data involving a mixture of various kinds of cells in which there may be dynamic changes in the cell population, are relatively meaningless with regard to concentration

My point is that there may be similar artifacts in the RNA to DNA ratio in situations such as this The finding that Dr Vilter reported is an increased RNA to DNA ratio in the marrows of acute leukemias The marrow contains a variety of cell types Some of them are relatively large cells with a large amount of cytoplasm and others are small cells with a minimal amount of cytoplasm

If in the course of the development of acute leukemia the marrow is largely *replaced by the immature forms these will be cells which are relatively rich in* cytoplasm which ha e a decreased nucleus to cytoplasmic olume ratio and as a general rule in so far as we are currently aware cells that are rich in cytoplasm tend to be rich in RNA also

We have no way of knowing at the moment just what this means but Dr Vilter s finding could well mean nothing more than the elaborate biochemical demonstration of the fact that the marrow of an acute leukemic patient is replaced by immature large cells This would be a rather expensi e way of doing what the microscope could do more cheaply

DR STUART C FINCH (New Haven Connecticut) I should like to make a comment about Dr Hamilton s paper on leukocyte survival

Dr Daniel Kline and I did some similar studies using $P^{32}$ instead of $C^{14}$ in normal subjects and in patients with polycythemia vera Our figures for blood survival corresponded very closely to those Dr Hamilton has reported They also were ery similar to those Dr Weisberger showed, with curves which were almost identical

In the three normal patients without hematologic disease and in the seven patients *with polycythemia vera the lymphocyte survival time was 8 to 9 days, and the* granulocyte peripheral blood survival time was 11 to 12 days

DR SVEN MOESCHLIN (Solothurn, Switzerland) I have been ery much interested in the work of Dr Weisberger with cysteine We have done work in the last two years trying to protect the leukopenic effect of benzene intoxication and we tried different cell type compounds It was shown as being completely negative *in effect, but the cysteine in guinea pig experimental benzene intoxication proved to*

39 Welsh H and Rittenberg D J Biol Chem 139 761 (1941)
40 White J and Edwards J E J Natl Cancer Inst 2 535 (1942)
41 White J White F R and Mider G B J Natl Cancer Inst 7 199 (1947)

# General Discussion

DR M TANNENBAUM (New York New York) I should like to relate if I may some of the results which have been presented today I think much of the data can be put into some sort of a uniform plan in the light of some of the recent findings which have been made in nucleic acid synthesis and also with proteolytic enzymes

Recently a study at New York University has shown that an enzyme polynucleotide phosphorylase is responsible for the synthesis or polymerization of various derivatives from ATP that is ADP and various other purine and pyrimidine bases which are linked to a ribose sugar and pyrophosphate This enzyme also catalyzes the reverse reaction Caspersson some time ago stated that there is a relationship between nucleic acids and protein synthesis Recently Gale at the Harvey Lectures stated that he has isolated from the derivatives of nucleic acids various factors which readily promote the incorporation of amino acids into proteins

It is possible that the function of this inhibitor which was mentioned this afternoon when it disappears allows the nucleic acid to promote the incorporation of various amino acids into protein This might possibly account for the increased sulfhydryl groupings in leukemic cells since these groupings according to some authors (Fruton E L Smith) have been shown to be associated with proteolytic enzymes which are now responsible for peptide synthesis as well

So there might be some correlation between the disappearance of the inhibitor and the observed increase in respiration which may be the source of these various types of purine and pyrimidine derivatives These derivatives are the raw materials necessary for the synthesis of nucleic acids which in turn might then promote the synthesis of proteins

DR NATHANIEL B KURNICK (Long Beach, California) As Dr Vilter has pointed out there are at least two inhibitors of DNA in mammalian tissues The principal *difference that I should like to stress between them is the species specificity* in the one Dr Vilter has studied and the one that Henstell and Freedman have studied

This difference in species specificity permits Dr Vilter using bovine pancreas as his source of enzyme to find a reduction in inhibitor content in leukemia and at the same time permits us using human serum as the source of the enzyme to find an increase in the inhibitor in leukemic cells

It seems possible that DNAase functions both in depolymerization and in DNA synthesis Perhaps the inhibitor which we have studied functions by inhibiting the depolymerization whereas the inhibitor which Dr Vilter has reported on inhibits synthesis This then might reconcile the apparent paradox in one going up and the other going down in a leukemic cell

DR L D HAMILTON (New York New York) I should like to correct a statement about DNAase I dont think there is any biochemical evidence that DNAase can synthesize DNA I believe we have to be cautious about saying that enzymes can work in both directions

I also should like to comment on the RNA and the base composition of RNA and the possible differences one may encounter between normal and leukemic cells I want to emphasize as I did at the beginning of my talk that there are many RNAases There are nuclear RNAases and there are several cytoplasmic RNAases and the differences we find between the normal and the leukemic RNA may represent differences that are relative shifts in the relative type of RNA rather than something specific between the normal and leukemic cell

As for Dr Bierman's comment we have no mathematical formulation for the rate of cell production at this time I am not clear as to what he meant by the fact that the white count had diminished toward the end of the experiment. Naturally during the period that we reported today on this patient with chronic lymphatic leukemia the white count, as mentioned by peripheral blood count, fluctuated and yet we saw no such fluctuation in the specific activity of the isolated purines from the cells

DR A S WEISBERGER (Cleveland Ohio) I just want to make a brief comment about this matter of life span The data are in good general agreement that the normal life span is somewhere in the neighborhood of 8 or 10 days and also that the lymphocytes have a very long life span

Our data would indicate that the chronic myeloid leukemia has a life span pretty much of the same order as that of the normal leukocyte but the one thing that has not been brought out is that of the acute leukemia Our data would indicate that the life span of acute leukemic leukocytes is extremely short compared to the others—somewhere in the neighborhood of 2 or 3 days

inhibit the leukopenia and also the anemia to some extent so I think cysteine may prove to be a very interesting problem in the future also in leukemia as Dr Weisberger mentioned

I was very glad to hear what Dr Hamilton said on the lifetime of leukocytes and also especially for leukocytes in chronic myeloid leukemia It may be that the longer lifetime of myelocytes in chronic myeloid leukemia is due not only to a different behavior in the lifetime of the cell in a real way but perhaps these cells can be used for the destruction of bacteria And so because we see that they do not have the normal ability to phagocytize in the body perhaps they live longer because they are not normal cells

Our findings in 1943 were another experimental way of studying the leukocyte ripening time The ripening time of the granulocyte was 6 to 8 days This fits together very well with the work of Dr Hamilton We calculated the lifetime of the leukocyte as 2 or 3 days It was the same as Osgood found

The division time of the myelocytes has been estimated by other investigators to be 40 to 60 minutes in cultures So from these one can calculate nearly exactly the total amount of leukocytes produced in one day and it is very interesting to see that all these things today fit together very well Also the findings with isotopes in Berkeley by the Lawrence group

DR HOWARD R BIERMAN (Duarte California) I want to congratulate Dr Hamilton on a very excellent paper

The increased life span of the lymphocyte in chronic lymphocytic leukemia would seem to suggest that there was an accumulation of cells in this patient rather than excessive production

If lymphocytes were made at or above the normal rate and were living for a longer period than normal one would expect a progressive increase in number of leukocytes in the intravascular spaces and in the tissues yet the chart demonstrated that in this patient with chronic lymphocytic leukemia the number of lymphocytes at the end of the study was the same or perhaps less than at the beginning

DR R W VILTER (Cincinnati Ohio) I really can say that I am in agreement with all the comments that have been made

In regard to Dr Kurnick s suggestion I think it is quite true that DNAase has to do with both the breakdown and the synthesis of DNA Whether these inhibitors which we are working with have anything to do with intracellular metabolism I don t know Perhaps the theory that Dr Kurnick has proposed may turn out to be correct but at the present time we will have to leave it in abeyance Nevertheless all these enzymes do control reversible reactions and both synthesize and degrade compounds

As far as the costly method of determining RNA is concerned I also agree with that One can look into the microscope and see that these cells do contain more RNA than the average bone marrow cell On the other hand the purpose in performing these tests is not to determine what we can see in the microscope but something which I could not report on today because we still don t know about it and that is whether or not the relationship of the various purines and pyrimidines to RNA and to DNA in the leukemic state may be different from that in the normal state That would be something that could not be determined except by these methods What I have shown so far are merely preliminary results

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## Part VII

# Further Biochemical Considerations in the Leukemias

Chairmen

C. A. Doan
College of Medicine
Ohio State University
Columbus, Ohio

Sven Moeschlin
Medical Department
Burgerspital
Solothurn, Switzerland

# 26

# Immunologic Studies of Protein Synthesis in Leukemia

HOWARD C HOPPS
School of Medicine of the University of Oklahoma
Oklahoma City Oklahoma

I must begin my presentation with an apology thus violating an established principle of effective propaganda The studies which our group are conducting are in their initial stages—the number of cases studied is too small to draw valid conclusions and I shall draw none On the positive side however some of the findings are provocative and suggest that our relatively new technique of approach to this problem may have considerable merit In my discussion I shall dwell more on the method than the results and hope that you may find something applicable to your own special problems

The principal change in plasma proteins relating to cancer so far as has been determined concerns increased levels of glycoprotein associated with albumin and all the globulin fractions In the case of the albumin associated polysaccharide part of this appears to be due to a mixture with seromucoid

The hypoalbuminemia so very often associated with cancer and demonstrated by a variety of methods including salt fractionation electrophoresis thermal coagulation polarography and the binding of certain dyes does not appear to be explainable simply by dietary deficiency or by abnormal loss of protein This hypoalbuminemia seems to be principally a quantitative effect but there may be qualitative aspects to it also

Greenstein (1) has commented "A vast literature exists on all phases which are known of the plasma proteins and yet it is interesting to contemplate that much of these almost countless studies rest upon two analytical procedures namely precipitation reactions involving sulfate salts and relative mobilities in an electric field (electrophoresis)"

One of the most sensitive means to detect specific proteins is an immunologic one and of course much work has been done with this method But until the last few years immunologic techniques at our disposal were most effective only when we had relatively pure homologous antigen The necessity for purity has been a tremendous handicap in analyzing such very complex antigenic mixtures as human plasma for example and yet it is just such complex mixtures as this that we must work with if we are to determine the presence of some new unique antigen mixed with the others—some new antigen that pertains to some specific disease e g leukemia Oudin (4) in 1946 made the first serious efforts to carry out analytical antigen antibody reactions in a gel whereby because of certain physical characteristics of the system multiple antigenic components of a mixture more or less sort themselves out to react individually with their corresponding specific antibodies Oudin coined the term immunochemical analysis" to denote the determination without resort to previous physicochemical fractionation of the number of antigens present in a mixture and their identification This important new development in method has opened a whole new area for immunologic study

For those unfamiliar with this method of Oudin I shall explain briefly (with oversimplification of the problems) that if one takes a tube of narrow bore say 2 to 3 mm and partially fills it with agar gel in which has been incorporated specific antibody and then overlays this with an antigenic mixture in solution components of the antigen will diffuse into the agar medium essentially unaffected by gravity or mechanical agitation Somewhat analogous to the separation of protein components that can be achieved by electrophoresis or ultracentrifugation or chromatography there occurs here a separation of antigenic components because of inherent physical characteristics leading to variation in the rate of diffusion Furthermore and this is a matter of crucial importance in this method *gradients* of concentration of the various antigenic components will be established in such a way that each will come to be in optimal proportions with the antibody that has been incorporated in the agar column At this region of optimal proportion there will develop a narrow band of maximal antigen antibody precipitation which will be readily visible Because (in most instances) antigen antibody precipitate is quite soluble in excess antigen as diffusion of antigen continues with increasing concentration the back end" of the precipitate of a given band is undergoing continuous progressive solution at the same time that the "front end" of the precipitate band is being added to or "built up" Therefore as the result of a continuous succession of

zones of optimal proportion the narrow band or zone of precipitation appears as though it were slowly migrating down the tube In the course of time (days) differences in rates of diffusion of antigenic components become more and more evident and bands of precipitate become "spread out" and distinctly separate from each other Certain conclusions can be reached by measuring the rate of migration and density of precipitate bands but I shall not go into these aspects of the reaction

Present interpretation of such gel precipitin reactions is based on several primary assumptions—for which there is strong support (*1*) As Oudin put it, "All antigens which do not cross react will behave independently i e as if each were present alone" (*2*) In a relatively simple diffusion system such as this there will be continuously decreasing concentration of antigen (external reactant) as one progresses from the liquid gel interface down into the gel Since the concentration of antibody within the gel remains *relatively* constant there can be only one region of optimal proportion per antigen within the agar column at any one time Hence there can be only one zone of antigen antibody precipitation for each antigen From these assumptions it follows that there will not be less antigenic components in the external reactant than the number of zones of antigen antibody precipitate developing in the column

There is a great deal that can be learned by such precipitin reactions in gels by this one dimensional tube method of Oudin With this technique identification of individual antigenic components can be carried out in several ways the most satisfactory of which is probably accomplished by pretreating the antiserum (before its incorporation into the agar column) with the antigen in question If reaction occurs the specific antibody will be "used up" and this will be evident by elimination of one of the zones of precipitation

Ouchterlony (4) in 1948 reported the first serious efforts to study antigen antibody reactions in gels by a system utilizing two dimensions in which plates were employed rather than tubes This relatively simple extension of the gel precipitation system described by Oudin greatly complicates precise analysis of just what is happening and the extent to which various factors influence the time location and amount of antigen antibody precipitation On the other hand it provides great advantage in certain types of study In this system antigen and antibody each in solution diffuse into a neutral area of reaction there establishing gradients of concentration which will include the zone of optimal proportions between antigen and antibody These "equivalence points" provide for antigen antibody precipitation in the form of relatively long lines rather

than narrow bands as in the tube method of Oudin Furthermore two samples of antigen can be set up to cross react with a single sample of antibody

Ouchterlony in his multiple diffusion two dimensional technique used a "plate of agar (contained within a petrie dish) in which small wells or reservoirs were made and filled with solutions of the antigen and antibody to be tested the majority of those who have used this method have employed similar reservoirs filled with antigen or antibody in solution One technical difficulty hard to overcome with this system is the gradual emptying of the reservoirs that occurs from diffusion so that they must be refilled during the course of the reaction When this is done there may develop opaque lines which are artifactual—probably related to temperature changes or sudden variations in concentration of the reactants Jennings and Malone (3) have presented a modification of Ouchterlony s method in which the reacting substances rather than being contained in solution in small reservoirs are incorporated into rather large volumes of agar and arranged so that each reactant presents a broad straight front from which it diffuses into a central area which contains only agar As he points out this arrangement is analogous to the usual Ouchterlony plate except that as the reagents diffuse out they form gradients with rectolinear concentration isobars rather than circular ones Jennings used a relatively small chamber the reaction area of which was an equilateral triangle 25 mm on each side Such an area is adequate to resolve lines of reaction if the system is not very complex In our studies however where numerous antigens and antibodies interact a larger reaction area is required if the individual lines are to be resolved The following is a description of the technique which we have employed

Equilateral triangular chambers were made from clear light weight plastic (Plexiglas) with inside dimensions of 120 mm on each side The chambers were 5 mm deep Each large chamber was then subdivided to form four smaller equilateral triangles of equal size by inserting strips of thin plastic (approximately 0 3 mm ) into narrow slots countersunk into the midpoint of each side of the large chamber (see Fig 1) Chambers were sterilized in a mixture of nitric and sulfuric acids after which they were washed with sterile distilled water

In preparation for a test the central triangle—i e the area of reaction —is filled with 1% agar containing 1 5000 merthiolate and this is allowed to solidify[1] The solutions of antigen and antisera which are to be

1 Methods of preparing agar etc were essentially those which have been described in detail by Oudin (4)

used are placed in a water bath and brought to a temperature of 50 Then they are mixed with 1% agar containing 1 5000 merthiolate (at the same temperature) in proportions of two parts antiserum or antigen solution to one part agar These mixtures are placed in the three triangular areas peripheral to the central agar filled area (area of reaction) and allowed to cool for 3 to 5 minutes after which a sterile razor blade is used to separate the agar from each of the thin dividing plastic strips and the strips are removed with thumb forceps This is a rather critical

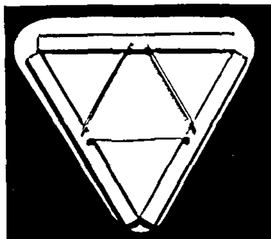


FIG 1 Photograph of the plastic "plate" used to secure two-dimensional antigen antibody precipitin reactions The central equilateral triangle is 60 mm on each side as are the three peripheral triangles The thin plastic strips which serve to divide the larger triangle into four smaller ones are removed once these four chambers are filled with agar antigen in agar or antibody in agar

procedure in that if the agar is too thin when the boundary strips are removed there will be intermixture if the agar has set too hard the interface will be torn After the strip is removed if the agar is of the proper consistency (i e temperature) the interface between the central and the peripheral triangles closes to a narrow relatively straight line Thus filled the chambers are placed in airtight (sealed) containers which include a small amount of water to saturate the atmosphere and prevent drying The containers are then placed in a constant temperature box Although the temperature at which the system is maintained will influence the speed of reaction this is not an important consideration within that range ordinarily referred to as room temperature The important thing is that there should be no sudden variations in tempera

than narrow bands as in the tube method of Oudin Furthermore two samples of antigen can be set up to cross react with a single sample of antibody

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[1] Methods of preparing agar etc were essentially those which have been described in detail by Oudin (4)

reaction Thus equivalents with the second, more dilute antibody by virtue of a second specific determinate group might lead to the formation of a second Oudin zone" If this is true (as Jennings pointed out) it seems that it would be much less likely to occur with a rapid precipitin system such as that afforded by rabbit antiserum since in such a case precipitates may be produced within a few minutes whereas horse antitoxin systems may require several hours to accomplish the same results

It would be enormously difficult to express precisely mathematically the relationship of those many factors which interact to influence the

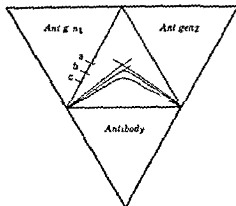



FIG 2 This schematic representation of precipitin reaction shows (a) independent reactions of antigen$_1$ and antigen$_2$ at the point of intersection indicating nonidentity" (b) partial fusion of the precipitate formed by antigen$_1$ and antigen (with antibody) at the point of intersection, indicating partial identity (c) complete fusion of the precipitate formed by antigen$_1$ and antigen (with antibody) at the point of intersection indicating identity

zone of precipitation and its density over a given time even if the exact concentration the physical characteristics of all the reacting antibodies and the amount and nature of the nonreacting components were known Of course we don t have this information in complex antigenic mixtures such as blood plasma Very fortunately we don t have to express mathematically the reaction we can observe in order to gain much useful information Without knowing *exactly* why we can get a good idea of the number of antigen antibody systems involved and often we can achieve a clear differentiation between those antigens which are in common and those which are not This method of Ouchterlony then provides a rather simple screening process by which means we may be able to identify unique antigenic components Once these are evident

ture In our laboratory we have fashioned a metal box with inner lining on all sides of approximately 4 inch Fiberglas batting and the plates are kept in there Because of the relatively large size of our reacting chamber (an equilateral triangle 60 mm on each side) and in consideration of the temperature of reaction (approximately 20 ) it requires five weeks for sufficient diffusion to occur so that the plates can be evaluated At the end of this time the results are recorded photographically

The important difference between the one dimensional and the two-dimensional systems which I have described is that in the latter both the antigen and antibody are diffusing into a "neutral zone whereas in the former (Oudin technique) the diffusion of one reactant (usually antigen) is into a region which contains a relatively fixed concentration of the other reactant (usually antibody) When *both* reactants are diffusing as in the plate method the point where the two reactants meet at optimal proportions is such that despite the passage of time the concentration of these reactants will remain in essentially the same *proportion* at the point where they first meet Thus individual points of precipitation remain essentially stationary with time Continued diffusion further out toward the center of the reaction area leads to the formation then of a succession of new points of maximal precipitation which form a *line* of precipitation

In a system where two samples of antigen are being compared in their reaction with a single antiserum our concern is with the behavior of lines of antigen antibody precipitate at their points of intersection Depending on this behavior we can classify the antigens as identical as closely related or as quite different (see Fig 2)

Wilson and Pringle (5) in their study on the interpretation of the Ouchterlony precipitin test, demonstrated that under ordinary conditions specific precipitates in the agar plate do not interfere with the free diffusion of unrelated antigens and antibodies They found further that identical antigens do always give rise to fusion of their specific precipitates Unrelated antigens occasionally gave fusion or partial intersection depending on relative concentration of the two precipitin systems According to their study false negative reactions may occur but not false positive reactions Jennings (2) however in a system employing whole immune horse serum and crude filtrate from tetanus cultures demonstrated what he believed to be formation of two zones by one antigen He hypothesized that "antigen after reacting with one antibody for a specific determinate group might still diffuse to form a gradient of concentrations beyond the line of visible precipitate from the first

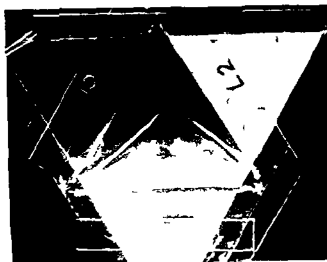



Fig 4 AC1 and C1 as in Fig 3 L2 = chronic granulocytic leukemic plasma The broad band like zones of precipitate conceal multiple individual lines of precipitate as is indicated on the C1 side



*Fig 5 AC1 and C1 as in Fig 3 L3 = chronic lymphocytic leukemic plasma* Note the shift (to left of midline) of the region of intersection which could be confused with a reaction of partial identity Such a shift indicates a significant difference in concentration of the antigenic components involved (C1 and L3) Here it appears that at least two antigenic components are of increased concentration in the leukemic plasma (L3) Note the lines of precipitate which have extended beyond the "area of reaction" down into the antibody gel region on the L3 side

we can apply procedures of absorption to test which specific fractions (prepared by salting out procedures electrophoresis chromatography etc ) are the ones in question

Figures 3 through 8 are photographs of antigen antibody precipitation in agar according to the method which has been described In this series high titer rabbit antiserum (A ) was produced to normal human plasma (C1) and plasma from patients with subacute chronic monocytic

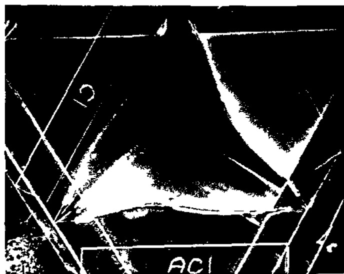



FIG 3 AC1 = rabbit antiserum (to normal human plasma) C1 = normal human plasma L1 = subacute chronic monocytic leukemia plasma Note at least six distinct linear zones of prec pitate A reaction of partial identity is indicated by ▼

leukemia (L1) chronic granulocytic leukemia (L2) chronic lymphocytic leukemia (L3) chronic granulocytic leukemia (L4) and acute monocytic leukemia (L5) None of these patients had received specific therapy for their leukemia prior to the time that the plasma sample was obtained

Rabbits were sensitized to the human plasma by multiple intramuscular "depot" injections of plasma adsorbed on $Al(OH)_3$ in addition to intravenous intraperitoneal subcutaneous and intradermal injections of plasma

Aliquot samples of antisera and plasma were frozen in plastic tubes and maintained at —20 until used The significance of the reactions depicted is given in the legends which accompany each of the figures

### Conclusions

I shall end much as I began—emphasizing that our study is in its early stages and that no firm conclusions can be drawn except perhaps that immunologic methods especially those in which precipitin reactions are developed in solid media seem to have an important application in the study of leukemia

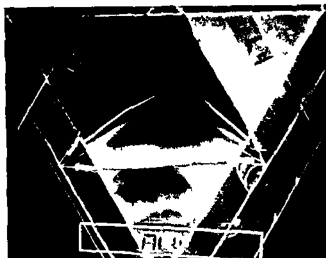



FIG 8 C1 as in Fig 3 L4 = chronic granulocytic leukemic plasma AL4 = rabbit antiserum against L4 plasma Intersections of C1-AL4 and L4-AL4 indicate identity however broad zones of precipitate may conceal reactions of "partial identity" Variations in concentration of antigenic components are indicated by a shift (to right) of some points of intersection and by a variation in width of precipitin zones

### Acknowledgments

I am pleased to acknowledge the very active collaboration of three senior medical students who have been working with me on these immunologic studies of protein synthesis in leukemia—Keith Klopfenstein Anthony Lynn and William Parker

### REFERENCES

1 Greenstein J P "Biochemistry of Cancer" 2nd ed Academic Press New York, 1954
2 Jennings R K, *J Bacteriol* 67 565 (1954)
3 Jennings R K, and Malone F *J Immunol* 72 411 (1954)
4 For discussion of development of these methods see Methods in Medical Research (A C Corcoran, ed ) Vol 5 The Year Book Publishers Chicago 1952
5 Wilson M W and Pringle B H *J Immunol* 75 460 (1955)

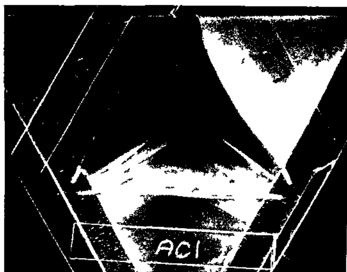



FIG 6 AC1 and C1 as in Fig 3 L5 = acute monocytic leukemic plasma Here linear zones of antigen antibody precipitate have extended well into the antibody gel region Though five weeks was allowed for reaction the two lines of precipitation farthest from the antibody source (AC1) have not intersected sufficiently to allow analysis One questionable reaction of partial identity may be seen

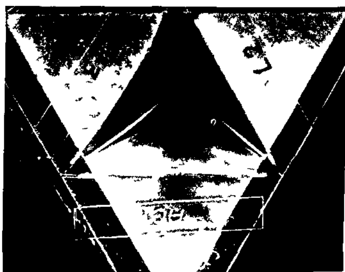


FIG 7 AC1 as in Fig 3 L2 = chronic granulocytic leukemic plasma L3 = chronic lymphocytic leukemic plasma The two leukemic plasma antigens give reactions of identity against antiserum to normal plasma

# 27

## Biochemical and Enzymatic Characteristics of Normal and Leukemic Leukocytes (with Particular Reference to *Leukocyte Alkaline Phosphatase*)[1]

WILLIAM N VALENTINE JAMES H FOLLETTE
DAVID H SOLOMON and JOHN REYNOLDS

Department of Medicine University of California Medical Center
and the Sawtelle Veterans Administration Center
Los Angeles California

Biochemical patterns in leukocytes from normal and leukemic subjects have now been studied from many different standpoints Glycolysis and respiration in intact cell and fortified homogenate systems the location of leukocyte histamine the enzymatic activities of $\beta$ glucuronidase transaminase acid and alkaline phosphatase esterase and lipase sulfhydryl constituents cell amino acid levels glycogen and many other nonenzymatic and enzymatic components of cells have been measured and analyzed by investigators in many areas It will not be the province of this report to review this broad subject or to document bibliographically the numerous investigations which have contributed to our knowledge of leukocyte constitution and metabolism Rather it will be confined to a discussion of leukocyte alkaline phosphatase with particular though not exclusive reference to its variations in leukemic and normal cells and its rather remarkable lability under the impact of disease The data reported represent largely the experiences of our laboratory over the last few years with quantitative studies of this intracellular enzyme For more comprehensive bibliographical reviews summarizing earlier work in this area including the development of the histochemical alkaline phosphatase technique by Gomori (3) its important applications in hematology by Wachstein (10) and others (2 6) the work of

[1] The work reported here has been supported by grants from Parke Davis and Company the United States Public Health Service and the Gladys F Bowyer Fund

Haight and Rossiter (4) and the recent extensive studies by Moloney and his co workers (5 11) the reader is referred to the original reports and to the comprehensive bibliographic material contained therein

In any general consideration of leukocyte biochemical constitution at least two important principles must be kept in mind First *morphologically different* leukocyte types such as the polymorphonuclear leukocyte and the lymphocyte can be expected to have different biochemical profiles in the *normal* subject by virtue of their inherent differences in structure and function Second *morphologically identical* cell types may have different biochemical activities in different disease states The latter situation is readily apparent in the case of leukocyte alkaline phosphatase

Leukocyte alkaline phosphatase is a phosphomonoesterase with pH optimum approximating 9 9 in human leukocytes In leukocytes it shares the properties of alkaline phosphatase as found ubiquitously in many tissues of the body requiring a metal activator such as $Mg^{++}$ and being markedly inhibited by certain sulfhydryl compounds such as cysteine and glutathione Its level in the leukocytes bears no discernible relationship to serum alkaline phosphatase and it appears to be a cytoplasmic component In the studies here reported its activity has been measured in incubation systems employing for the most part sodium $\beta$ glycero phosphate as substrate saponin as a membrane damaging agent facilitating enzyme substrate contact a buffered media at pH 9 9 a 1 hour incubation at 37 and a final spectrophotometric measurement of inorganic phosphorus liberated

Initial studies have shown the following features of alkaline phosphatase activity in separated human leukocytes prepared as previously described (*1*) Expressed as milligrams of phosphorus liberated per hour per $10^{10}$ cells from substrate under the incubation conditions given above unit cell leukocyte phosphatase is often increased severalfold in *a wide variety of disease states It is for example markedly increased* in such diverse pathologic states as infection trauma diabetic acidosis acute gout intestinal hemorrhage cerebral vascular accidents acute urinary retention and myocardial infarction (7 9) These may have multiple features in common but an obvious common denominator is that in all an increase in pituitary adrenal activity would be expected (2) In man alkaline phosphatase activity is low or absent in blast cells and mononuclear leukocytes (1 10 11) In many of the conditions where unit leukocyte activity is high some degree of polymorphonuclear leukocytosis pertains The elevations in phosphatase activity however which are often three or fourfold above normal values and sometimes

much greater cannot be explained in terms of changes in the differential formula of the cell population analyzed (3) The granulocytes of chronic myelocytic leukemia characteristically exhibit low alkaline phosphatase activity (8 11) and this is the usual situation over wide variations in the differential formula of cell populations analyzed in this disease including those in which the leukocytes are almost exclusively mature and the total leukocyte count temporarily approximates the normal range On the other hand in polycythemia rubra vera with leukemoid features and morphological cell populations mimicking those of chronic myelocytic leukemia and in many nonleukemic myeloproliferative states with circulating cell spectra simulating chronic myelocytic leukemia unit leukocyte phosphatase is often well above the normal range and manyfold greater than that found in this form of leukemia. This points up a difference not attributable to maturity or cell type In one group of seventeen cases of polycythemia vera with leukemoid features of this type average values were twentyfold greater than those in a series of chronic myelocytic leukemia (8) (4) For reasons which are not clear most cases of *uncomplicated* but active rheumatoid arthritis many cases of *uncomplicated* rheumatic fever and cases of disseminated lupus erythematosus fail to show elevated leukocyte alkaline phosphatase values (9) This would appear to indicate that active disease does not universally evoke the leukocyte phosphatase elevation and that for some reason this failure is evident in many cases of active so called collagen diseases Figure 1 summarizes data bearing on many of the points made above

The findings of marked elevations in this intracellular enzyme in diverse clinical disease states naturally raised the question of whether increased adrenal corticosteroid activity might not be at least one of the responsible mediators of the phenomenon This has indeed been found to be the case (9 also our unpublished data manuscript in preparation) Substantial doses of ACTH or 17 OH corticosteroids administered over a 72 hour period result in an average approximately threefold increase in unit leukocyte phosphatase activity sometimes somewhat less and sometimes somewhat more This applies when these agents are administered to subjects whose pretreatment phosphatase values are within the normal range and not necessarily in pathologic states where levels are already substantially elevated In the latter situations a further response to exogenous ACTH or 17 OH corticosteroids *may or may not* occur In an occasional ostensibly normal subject this customary response has not been evoked a fact which is unexplained but possibly related to anomalies of individual response inadequate blood levels or

picture Prior to therapy unit leukocyte alkaline phosphatase activity was very low and no elevation was elicited by either ACTH or hydrocortisone The patient was placed on Myleran and has obtained a complete and unusually long remission which has now persisted without any

TABLE I

A COMPARISON OF THE EFFECT OF ACTH HYDROCORTISONE AND OTHER AGENTS ON LEUKOCYTE ALKALINE PHOSPHATASE ACTIVITY

(The values given are averages)

| Agent | Dosage schedule | Alkaline phosphatase before Rx | Alkaline phosphatase after 72 hr of Rx |
|---|---|---|---|
| Gel alone | 1 ml q 8 hr × 9 I M | 28 0 | 38.5 |
| ACTH gel | 10-20 units q 8 hr × 9 I M | 30 0 | 64 0 |
| ACTH gel | 30-40 units q 8 hr × 9 I M | 23 5 | 60 0 |
| Hydrocortisone | 40-50 mg q 8 hr × 9 P O | 35 0 | 58 0 |
| Hydrocortisone | 60-70 mg q 8 hr × 9 P O | 24 5 | 57 5 |
| DOCA | 6 5 mg q 8 hr × 9 I M | 25 5 | 25 0 |
| Ascorbic acid | 500 mg q 12 hr × 6 I M | 30 0 | 27 0 |

Expressed as milligrams of phosphorus liberated per hour by $10^{10}$ leukocytes from the substrate sodium $\beta$ glycerophosphate at 37

TABLE II

EFFECT OF ACTH GEL ON LEUKOCYTE ALKALINE PHOSPHATASE ACTIVITY† IN THREE SUBJECTS WITH ADDISON S DISEASE AND THREE SUBJECTS WITH PANHYPOPITUITARISM

| Disease | Case number | Alkaline phosphatase† before Rx | Alkaline phosphatase† after 24 hr of Rx | Alkaline phosphatase† after 72 hr of Rx |
|---|---|---|---|---|
| Addison s disease | 1 | 16 0 | 25 0 | 20 0 |
| | 2 | 22.5 | 31 0 | 32 0 |
| | 3 | 48.5 | 37 5 | 38 0 |
| | Average | 29 0 | 31 2 | 30 0 |
| Panhypopituitarism | 1 | 22.5 | 44 0 | 82 5 |
| | 2 | 28 0 | 71 0 | 104 0 |
| | 3 | 37 0 | 44 0 | 106 0 |
| | Average | 29.2 | 53 0 | 97.5 |

Administered in amount of 40 units intramuscularly every 8 hours for 72 hours

† Expressed as milligrams of phosphorus liberated per hour by $10^{10}$ leukocytes from the substrate sodium $\beta$ glycerophosphate at 37

TABLE III

EFFECT OF ACTH GEL AND HYDROCORTISONE ON LEUKOCYTE ALKALINE PHOSPHATASE ACTIVITY IN FIVE SUBJECTS WITH CHRONIC MYELOCYTIC LEUKEMIA AS COMPARED WITH SEVEN NONLEUKEMIC SUBJECTS†
(Values given are averages)

| Subjects | Dosage schedule | Alkaline phosphatase before Rx | Alkaline phosphatase after 24 hr of Rx | Alkaline phosphatase after 72 hr of Rx | Alkaline phosphatase 2–7 days after Rx stopped |
|---|---|---|---|---|---|
| Nonleukemic | ACTH gel 40 units q 8 hr | [illegible]8 0 | 42 0 | 74 0 | 23 0 |
| Chronic myelocytic leukemia | I M for 72 hr | 8 0 | 9 5 | 7 5 | 7 0 |
| Nonleukemic | Hydrocortisone 60–70 mgs q 8 hr | 20 0 | 38 0 | 50 0 | 33 0 |
| Chronic myelocytic leukemia | P O for 72 hr | 5 0 | 6 5 | 7.5 | 5 0 |

Expressed as milligrams of phosphorus liberated per hour by $10^{10}$ leukocytes from the substrate sodium β glycerophosphate at 37

† Data on two exceptional cases of chronic myelocytic leukemia in remission discussed fully in text are not included in the above compilation

picture Prior to therapy unit leukocyte alkaline phosphatase activity was very low and no elevation was elicited by either ACTH or hydro cortisone The patient was placed on Myleran and has obtained a complete and unusually long remission which has now persisted without any

TABLE I

A Comparison of the Effect of ACTH Hydrocortisone and Other Agents on Leukocyte Alkaline Phosphatase Activity

(The values given are averages)

| Agent | Dosage schedule | Alkaline phosphatase before Rx | Alkaline phosphatase* after 72 hr of Rx |
|---|---|---|---|
| Gel alone | 1 ml q 8 hr × 9 I M | 28 0 | 38.5 |
| ACTH gel | 10–20 units q 8 hr × 9 I M | 30 0 | 64 0 |
| ACTH gel | 30–40 units q 8 hr × 9 I M | 23 5 | 80 0 |
| Hydrocortisone | 40–50 mg q 8 hr × 9 P O | 35 0 | 58 0 |
| Hydrocortisone | 60–70 mg q 8 hr × 9 P O | 24 5 | 57 5 |
| DOCA | 6 5 mg q 8 hr × 9 I M | 25 5 | 25 0 |
| Ascorbic acid | 500 mg q 12 hr × 6 I M | 30 0 | 27 0 |

Expressed as milligrams of phosphorus liberated per hour by $10^{10}$ leukocytes from the substrate sodium β glycerophosphate at 37

TABLE II

Effect of ACTH Gel on Leukocyte Alkaline Phosphatase Activity† in Three Subjects with Addison s Disease and Three Subjects with Panhypopituitarism

| Disease | Case number | Alkaline phosphatase† before Rx | Alkaline phosphatase† after 24 hr of Rx | Alkaline phosphatase† after 72 hr of Rx |
|---|---|---|---|---|
| Addison s disease | 1 | 16 0 | 25 0 | 20 0 |
| | 2 | 22.5 | 31 0 | 32 0 |
| | 3 | 48 5 | 37 5 | 38 0 |
| | Average | 29 0 | 31 2 | 30 0 |
| Panhypopituitarism | 1 | 22 5 | 44 0 | 82 5 |
| | 2 | 28 0 | 71 0 | 104.0 |
| | 3 | 37 0 | 44 0 | 106 0 |
| | Average | 29 2 | 53 0 | 97 5 |

Administered in amount of 40 units intramuscularly every 8 hours for 72 hours

† Expressed as milligrams of phosphorus liberated per hour by $10^{10}$ leukocytes from the substrate sodium β glycerophosphate at 37

## REFERENCES

1 Beck W S and Valentine W N *J Lab Clin Med* **38** 245 (1951)
2 Dempsey E W and Wislocki G B *Physiol Revs* **26** 1 (1946)
3 Gomori G *Proc Soc Exptl Biol Med* **42** 23 (1939)
4 Haight W F and Rossiter R J *Blood* **5** 267 (1950)
5 Moloney W C and Lange R D *Blood* **9** 663 (1954)
6 Rheingold J J and Wislocki G B *Blood* **3** 641 (1948)
7 Valentine W N and Beck W S *J Lab Clin Med* **38** 39 (1951)
8 Valentine W N Beck W S Follette J H Mills H and Lawrence J S *Blood* **7** 959 (1952)
9 Valentine W N Follette J H Hardin E B Beck W S and Lawrence J S *J Lab Clin Med* **44** 219 (1954)
10 Wachstein M *J Lab Clin Med* **31** 1 (1946)
11 Wiltshaw E and Moloney W C *Blood* **10** 1120 (1955)

form of therapy for eighteen months The spleen is impalpable the myeloid/erythroid ratio in bone marrow normal and the total and differential leukocyte count normal with basophils now only 1 to 2% It is not known whether this represents one of the rare spontaneous remissions superimposed on a therapeutic remission or not In any event unit cell phosphatase activity has returned to normal and refractoriness to ACTH and hydrocortisone is no longer present It should be emphasized again at this juncture that this is not the usual experience in therapeutic remission but rather the exception The data however raise some interesting philosophical questions They indicate that an occasional individual early in the course of the disease and in the presence of very good remissions may show a reversion to normal phosphatase pattern and normal responsiveness to ACTH and 17 OH corticosteroids It is not known whether leukemia of this type originates as some over all metabolic transformation or whether loci of abnormal leukemic cells first appear and then overgrow and suppress normal cell potential If the situation involves over all transformation the data in these two cases indicate that an occasional essentially complete remission may be accompanied by a more normal metabolic pattern at least in respect to the phosphatase aberration If the two population or "two cell line ages hypothesis is adopted the data suggest that an occasional exceptional remission may be accompanied by reassertion of the normal cell lineage temporarily If this were the case reversion of the phosphatase pattern would be due actually to the fact that during the remission normal and not leukemic cells were being analyzed The failure to obtain reversion in most therapeutic remissions does not favor either possibility since remission might be associated in most instances not with reascendency of the hypothetical normal cell line but only by partial repression of the leukemic strain These speculations are of course of a philosophical nature at the moment and the authors express no brief for either hypothesis in terms of existent data It is hoped that experimental situations may be found to test these two possibilities more satisfactorily and that biochemical differences between "normal and leukemic" cells offer some hope that this may ultimately be susceptible to testing

## Acknowledgments

The authors gratefully acknowledge the technical assistance of Mr Peter Sugarman Mrs Estelle Wong Mrs Carol Levin and Miss Elaine Levi in the investigations reported

# 28

## Metabolism of Folic Acid and Citrovorum Factor in Leukemic Cells[1]

R R ELLISON and D J HUTCHISON

Chemotherapy Service Memorial Center for Cancer and Allied Diseases and the Divisions of Experimental and Clinical Chemotherapy Sloan Kettering Institute New York New York

The treatment of acute leukemia with folic acid antagonists has severe limitations two thirds of the children so treated do not develop complete remissions the great majority of adults fail to respond and all patients whose disease is originally sensitive to these agents ultimately relapse despite continuance of therapy

The folic acid antagonists are thought to interfere with folic acid and citrovorum factor (CF) metabolism at at least three sites the conversion of conjugated folic acid to available folic acid the conversion of folic acid to CF and the conversion of CF to a postulated more active form In so doing the folic acid antagonists presumably inhibit cell growth by blocking utilization of the one carbon fragments necessary for synthesis of nucleic acids and such amino acids as methionine and serine

Swendseid *et al* (9) reported an increase in the content of CF in leukemic cells which they felt was correlated with the degree of cell immaturity the highest values being found in acute leukemia No correlation was made between the CF content and the later response of these patients to folic acid antagonists

Broquist *et al* (2) found that a mutant strain of *S faecalis* resistant to amethopterin (4 amino 10 methylpteroylglutamic acid) (Methotrexate) was far more efficient in forming CF from folic acid than was the wild

[1] These studies were supported by research grants C1689 and C679 from the National Cancer Institute of the National Institutes of Health Public Health Service and institutional grants from the American Cancer Society the Damon Runyon Memorial Fund for Cancer Research the Lasker Foundation and the Black Stevenson Foundation

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strain. Work by Hutchison on the liberation of CF and the formation of CF from folic acid by leukemic mouse spleen cells (6) indicated that an amethopterin sensitive strain (line I) was slightly more effective than the resistant strain (line I/A) in the formation of CF from folic acid. Line I/A leukemic cells, however, always contained more free CF prior to incubation *in vitro* and during the first 5½ hours of incubation. Amethopterin had less effect on both these enzymatic reactions in line I/A. Spleens from line I leukemia contained three times the amount and spleens from line I/A leukemia four times the amount of total CF found in the normal mouse spleens (this was calculated as millimicrograms of CF per milligram dry weight).

Clinical sensitivity to folic acid antagonists in acute leukemia may indicate a greater dependence on CF for growth in the sensitive leukemic leukocytes than in the resistant cell or normal leukocytes. This could be a reflection of inability to form CF from folic acid adequately, inability to free CF from the conjugated form, or inability to utilize the CF properly when present. If this greater need for CF exists, it might be possible to demonstrate that a leukemic patient sensitive to the folic acid antagonists would excrete a lower percentage of a given dose of CF than would a resistant patient. Investigation of the serum levels and urinary excretion of amethopterin and CF after administration of these compounds to patients with acute leukemia showed no difference, however, between the values obtained with those subsequently sensitive to amethopterin therapy and those resistant to it (Ellison, Murphy, Hutchison and Burchenal, unpublished observations). It was thought, therefore, that study of the development of resistance in human acute leukemia should involve investigation of intracellular rather than extracellular factors.

This report will deal primarily with studies of the CF content of leukemic and normal leukocytes. Mention will be made of the effects thereupon of amethopterin *in vivo* and the rate of liberation of free CF from the conjugated form. The finding of thymidine in the incubated cell suspensions will also be discussed.

## Methods

In the work to be reported, all glassware which came in contact with the leukocytes had been previously siliconed. We routinely drew 20 ml of blood. Ethylenediaminetetraacetic acid was used as anticoagulant and dextran was employed to sediment the erythrocytes rapidly. After the mixture stood at room temperature for 2 to 3 minutes, all procedures were carried out in the cold. After the erythrocytes sedimented (about

45 minutes) the plasma and white cell mixture was removed and centrifuged lightly. The sedimented leukocytes were resuspended in 1% acetate buffer pH 4.5 made up in isotonic saline. At this pH CF conjugase activity is maximal (9). Cell counts were done on the white cell suspension so that CF could be expressed as the amount per cell. Aliquots of the suspension were incubated for variable periods of time at 37° in the presence of ascorbic acid. Twenty hours was considered (by analogy with bacterial studies) the necessary incubation period for the liberation of the total amount of CF. Further enzymatic action was then prevented by boiling. This also served to lyse the leukocytes. After the addition of bicarbonate to produce a pH of 7.0 and dilution of the sample it was autoclaved (7) and centrifuged. Assays were done on the supernatant.

Assays for CF were done with *Pediococcus cerevisiae* (5). The sample was further diluted to at least a tenfold dilution of the original white cell suspension to prevent inhibition of the assay organism by dextran or EDTA. With this assay as little as 0.1 mγ of CF per milliliter of sample or 1 mγ of CF per milliliter of white cell suspension could be determined. The lower limits of the determination therefore depended on the white blood cells available as well as on the assay procedure. Thus with a cell count in the suspension of 20 000/mm$^3$ we could measure a minimum of 50 mγ × $10^{9}$ per single leukocyte but with a count of 100 000 as little as 10 mγ × $10^{9}$ per leukocyte could be determined.

In cases where the patient had been receiving amethopterin or this substance had been added *in vitro* to the cell suspension the assay was performed with a mutant *P. cerevisiae* resistant to amethopterin. The dose response curve of this organism with CF is identical to that of the wild strain. The response of the sensitive and resistant organisms to thymidine was found to differ however. This will be discussed later.

Thymidine assays on the incubated cell suspensions were also performed microbiologically with *L. arabinosus* in a sulfanilamide medium. Under these conditions CF in the amounts present in the cells does not inhibit or enhance the growth of the organism. The lower limit of the assay was 1.0 γ of thymidine per milliliter of cell suspension when a minimal dilution of 1:10 was used.

## Results

All results to be presented were obtained in adults. All CF values mentioned are in terms of mγ × $10^{9}$ per single leukocyte. In references to CF in this report the amount in the cells has been referred to a syn

thetic leucovorin standard The results would have to be halved to give the actual natural CF content since the synthetic material is a racemic mixture

Figure 1 shows the data obtained on total CF content The closed circles indicate those patients with no recent amethopterin therapy the open circles those receiving amethopterin Considering first only the former the range in the normals was 50 to 200 Patients with chronic lymphatic leukemia in relapse had CF values in the same range with

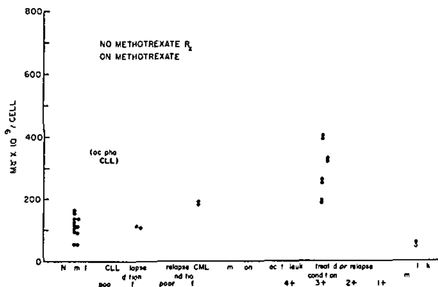



FIG 1 Total CF content of human leukocytes

the exception of one patient who was in an acute phase morphologically and clinically and died several days later Patients with chronic granulocytic leukemia in relapse and one chronic granulocytic leukemic in remission also had similar levels Three assays on two patients with chronic granulocytic leukemia who were in very poor condition but were not in a blastic phase were elevated We have attempted to subdivide the group with acute leukemia in relapse or untreated on the basis of clinical condition at the time of the test 4+ indicates those who died within 2 weeks of the test 3+ those who appeared equally ill but did not die in that period and 2+ and 1+ those who were in better condition clinically There was no difference among the first three of these groups They ranged from 90 to 800 The fourth group was not large enough to compare The levels in patients in complete hema

tological and clinical remission resembled the normals In the two cases where assays were obtained before and during remission the remission levels were lower

Those values obtained on patients receiving amethopterin and assayed on the resistant organism were all quite low It was thought that this might represent an *in vivo* effect of the drug such as occurred in leukemic mouse spleens

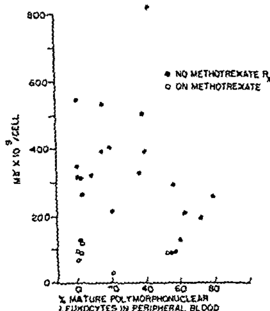



FIG . Total CF content of leukocytes of acute leukemia in relapse or untreated

Figure 2 shows an attempt to correlate the apparent level of total CF in acute leukemia in relapse or untreated with the per cent of mature polys in the peripheral blood There was no correlation There was also no correlation between the total CF and the peripheral white count or the white count in the cell suspension used or the subsequent development of remission or the duration of life after the time the test was performed As already noted there was no correlation between the total CF and the clinical condition when the patient was in relapse

Figure 3 shows typical curves illustrating the rate of liberation of CF from the conjugated form *in vitro* The rate difference noted in chronic lymphatic leukemia has been seen in all cases studied We have three

other curves similar to the one shown on a normal subject (representing two individuals)

Recently we noted that the assays gave a peculiar pattern with the various dilutions of sample used When read from the standard CF curve the most dilute sample almost always seemed to contain more

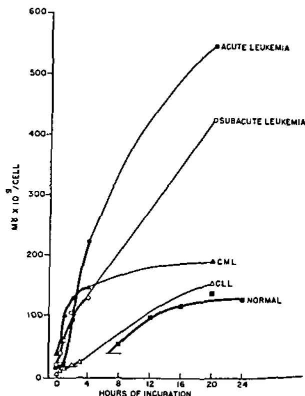



FIG 3 Liberation of free CF by human leukocytes

CF than did the other three of the assay We therefore looked for some other substance which might be acting as a growth factor for *P cerevisiae* By assay with *L arabinosus* in a sulfanilamide medium thymidine was identified The presence of this substance was verified chromatographically Figures 4 and 5 show the appearance of the dose response curves when various mixtures of thymidine and CF are used in the standard assay with *P cerevisiae* Thymidine assays have been run on some of the available samples Thymidine was not detectable in cell suspensions incubated less than 12 hours Thymidine levels of 120 to

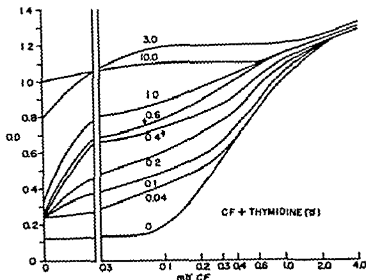



Fig. 4 The sparing effect of thymidine on the response of *Pediococcus cerevisiae* to citrovorum factor

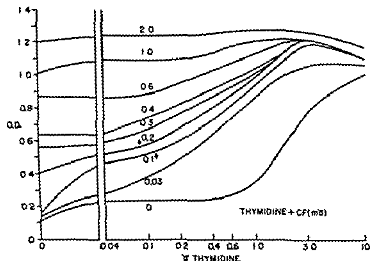



Fig. 5 The sparing effect of citrovorum factor on the response of *Pediococcus cerevisiae* to thymidine

550 $\mu\mu g \times 10^{9}$ per cell were found in cell suspensions incubated 20 hours In the relatively few assays done there was no obvious correlation between thymidine level and the apparent CF activity of the cells

We then found that although the amethopterin sensitive and resistant *P cerevisiae* strains show the same response to CF the resistant strain does not respond to thymidine (Fig 6) This may in part account for the uniformly low apparent values for CF found in patients who had been receiving amethopterin and whose assay therefore was done with the resistant strain Accordingly reassays were done on the cell suspen

TABLE I

TOTAL APPARENT CF CONTENT OF HUMAN LEUKOCYTES

| Clinical status | Assay organism | |
|---|---|---|
| | *P cerevisiae* | *P cerevisiae/A* |
| Normal | 208 | 104 |
| | 126 | 51 |
| | 179 | 70 |
| | 181 | 94 |
| Hodgkin s disease with infection | 108 | 32 |
| Chronic granulocytic leukemia | 312 | 96 |
| | 172 | 40 |
| Acute leukemia | 212 | 167 |
| | 193 | 100 |
| | 390 | 159 |
| | 676 | 227 |
| | 569 | 287 |

sions of several people who had not received the antimetabolite Figure 7 shows the results with the cells from a patient with chronic granulocytic leukemia in the terminal phase and indicates that in the cells that had been incubated 20 hours only one third of the apparent CF value actually represented CF Most other reassays showed similar results (Table I) Assays were done on one individual employing all three organisms From these results we attempted to estimate the thymidine content using the checkerboard graphs (Figs 4 and 5) The amount of thymidine found by this calculation came to one half of that actually found with *L arabinosus* assay This may indicate the presence of substances other than thymidine in the incubated cell suspensions which stimulate the growth of *L arabinosus*

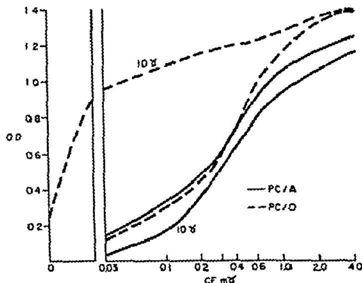



FIG 6 The response to citrovorum factor of amethopterin sensitive and resistant strains of *Pediococcus cerevisiae* in the presence and absence of 10 γ of thymidine

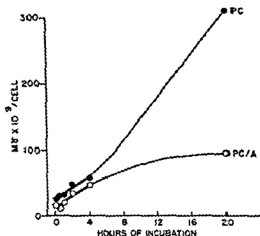



FIG 7 Liberation of free CF by human leukocytes One third of the apparent CF value actually represents CF

## Discussion

Although expressed in different terms our data on apparent CF content were in general agreement with those of Swendseid and her co workers Probably the correlation which she observed between cell type and CF activity was due to the absence in her series of cases of acute leukemia where large numbers of mature cells were present Since the same conditions of incubation were used with the two exceptions of addition of ascorbic acid and of autoclaving after incubation it also seems probable that the same mixture of growth substances was being measured but was called CF

Despite the problems of identification our data indicated however that in many adults with acute leukemia and in some acutely ill with chronic granulocytic or lymphatic leukemia the circulating leukocytes contained more than the normal amount of substances which promoted the growth of *P cerevisiae* Part of this is CF and part is thymidine Perhaps some other as yet unidentified substances are present Which of these is in fact elevated in the leukocytes cannot be ascertained from the data we now have

Studies of growth factors for *S faecalis* and *P cerevisiae* in marine algae and in lichens (1 3 4 8) indicate that treatment of such organisms with chicken pancreas or hog kidney homogenates caused the release of at least twelve factors These included pteroyltriglutamic acid pteroyldiglutamic acid $N^{10}$ formylpteroic acid $N^{10}$ formylfolic acid folinic acid (CF) thymidine folic acid $N^5$ formyltetrahydrotriglutamic acid and N formyltetrahydrodiglutamic acid The last two substances (as well as another unidentified compound) can stimulate the growth of *P cerevisiae* and are the first reported compounds other than CF and thymidine able to do so

The conditions used in treating the leukocytes undoubtedly caused autolysis of the cells accounting for the presence of thymidine Detectable amounts of thymidine were not present in cell suspensions incubated for less than 12 hours There is no reason to connect the appearance of thymidine with the reactions involved in the liberation of CF from the conjugated form

Despite the fact that we do not know what substance caused the increase in the apparent CF activity it is of interest that the presence of an increased amount of growth factor for *P cerevisiae* did not depend merely on the presence of blast cells This might mean that the entire series of granulocytes mature and immature in such patients is abnormal

**Summary**

1 The total CF content of human leukocytes was studied After incubation of the leukocytes to convert all conjugated CF to the free form assay with *P cerevisiae* indicated greater than normal apparent CF activity in the leukocytes in many of the cases of acute leukemia This was also found in some terminal cases of chronic granulocytic and lymphatic leukemia

2 The increase in apparent CF activity could not be correlated with the percentage of blast cells in the peripheral blood

3 Part of the apparent CF activity was found to be due to the presence of thymidine in the incubated cell suspensions

4 Thymidine does not stimulate growth of an amethopterin resistant strain of *P cerevisiae* In almost all the leukocyte assays done with both the amethopterin sensitive and resistant strains the apparent CF activity was lower with the latter

5 The exact nature of the increase in the total apparent CF activity of the leukocytes from acute leukemia remains to be defined

## REFERENCES

1 Bolinder A Widoff E and Ericson L E *Arkiv Kemi* 6 487 (1953)
2 Broquist H P Kohler A R Hutchison D J and Burchenal, J H *J Biol Chem* 202 59 (1953)
3 Ericson L E *Arkiv Kemi* 6 503 (1953)
4 Ericson L E Widoff E and Banhidi Z G *Acta Chem Scand* 7 374 (1953)
5 Felton E A and Niven C F *J Bacteriol* 65 481 (1953)
6 Hutchison D J and Burchenal J H "Chemistry and Biology of Pteridines J & A Churchill London 1954
7 Nichol C A Anton A H and Zakrzewski S F *Science* 121 275 (1955)
8 Sjostrom A C M and Ericson L E *Acta Chem Scand* 7 870 (1953)
9 Swendseid M E Bethell F H and Bird O D *Cancer Research* 11 864 (1951)

# Added Comment

## Labeling of RNA of Rabbit Peritoneal Exudate Polymorphonuclear Leukocytes[1]

R J ROSSITER

Department of Biochemistry University of Western Ontario
London Canada

*Interest* in the metabolism of the nucleic acids of leukocytes has been greatly stimulated by the basic studies of Thorell (21) who used the ultraviolet absorption technique of Caspersson (4) and by the later finding that many purine analogs and folic acid antagonists were able to cause a transient remission in both experimental and spontaneous leukemia A large number of these anticancer agents inhibit the formation of nucleic acids (19)

Of recent years the labeling of nucleic acids with radioactive precursors has proved a useful tool for the study of nucleic acid metabolism The present paper describes some of the metabolic potentialities of rabbit polymorphonuclear leukocytes in this regard The polymorphonuclear leukocytes were obtained from peritoneal exudates of the rabbit by the method of de Haan (9) The ability of these cells to label *ribonucleic acid (RNA) has been compared with that of rat liver slices* studied under similar conditions

### Results

Table I shows the specific activities of the RNA nucleotides of the rabbit polymorphonuclear leukocytes incubated for 2 hours in Krebs Ringer bicarbonate buffer pH 7 4 in the presence of inorganic $P^{3}$ After the incubation the RNA nucleotides were separated by electrophoresis on paper by the method of Davidson and Smellie (5) The nucleotides so isolated are mixtures of the *a* and *b* isomers (3) now known to be the 2 and 3 phosphoribosides respectively (2, 12) It

1 This work was aided by a grant from the National Cancer Institute of Canada

can be seen that all four nucleotides were labeled adenylic acid and uridylic acid being much more active than guanylic acid and cytidylic acid

In Table I are also given results for liver slices incubated with inorganic $P^{3}$ under similar conditions Again all four nucleotides were

TABLE I

LABELING OF RNA NUCLEOTIDES IN RABBIT POLYMORPHONUCLEAR LEUKOCYTES AND RAT LIVER SLICES INCUBATED WITH INORGANIC $P^{3}$ FOR 2 HOURS IN KREBS RINGER BICARBONATE BUFFER

| Nucleotide | Number of experiments | Specific activity (mean ± S E mean) counts/min /γ P Polymorphonuclear leukocyte | Liver slice |
|---|---|---|---|
| Adenylic acid | 6 | 42.2 ± 7 1 | 2 0 ± 2 5 |
| Guanylic acid | 6 | 5 2 ± 3 9 | 16.3 ± 0 9 |
| Cytidylic acid | 6 | 29.5 ± 4 6 | 19 0 ± 1 0 |
| Uridylic acid | 6 | 39 0 ± 8 1 | 3.2 ± 1 5 |

TABLE II

LABELING OF RNA BASES IN RABBIT POLYMORPHONUCLEAR LEUKOCYTES AND RAT LIVER SLICES INCUBATED WITH FORMATE-$C^{14}$ FOR 2 HOURS IN KREBS RINGER BICARBONATE BUFFER

| Base | Number of experiments | Specific activity (mean ± S E mean) counts/min /μmole Polymorphonuclear leukocyte | Liver slice |
|---|---|---|---|
| Adenine | 5 | 0 | 4050 ± 880 |
| Guanine | 5 | 0 | 630 ± 160 |
| Cytosine | 5 | 0 | 0 |
| Uracil | 5 | 0 | 0 |

labeled but for each nucleotide the activity was considerably less than that of the corresponding nucleotide of the leukocytes As found for the white cells the adenylic acid and uridylic acid were more active than the guanylic acid and the cytidylic acid thus confirming previous reports of *in vivo* studies (6 23) The same is apparently true for other tissues Adenylic acid and uridylic acid were found to be more active than guanylic acid and cytidylic acid in brain tissue *in vitro* (7) and in adrenal tissue *in vivo* (14)

Table II shows the specific activities of the bases of RNA when similar preparations of rabbit polymorphonuclear leukocytes and liver slices

were incubated with formate $C^{14}$ instead of inorganic $P^{32}$ as the source of the radioactivity In these experiments the RNA nucleotides were separated by the method of Schmidt and Thannhauser (18) The bases were liberated by perchloric acid hydrolysis and were then isolated by the paper chromatographic method of Wyatt (25) as described by Mannell and Rossiter (15)

It will be noted that with the liver slices good incorporation was observed for the RNA adenine with the guanine being less active As anticipated there was negligible activity in the two pyrimidines A surprising finding was the complete lack of labeling of both purines as well as pyrimidines in the RNA of the leukocytes

The RNA of the polymorphonuclear leukocytes is thus labeled from inorganic $P^{32}$ more actively than the RNA of liver slices but unlike the RNA of liver slices it is not labeled from formate $C^{14}$

## Discussion

The mature rabbit polymorphonuclear cell from peritoneal exudates would appear to lack the enzyme equipment necessary for the formation of RNA *in vitro* from single carbon units This is in sharp contrast to the findings for spleen (10 15) bone marrow (1 22) mouse leukemia cells (24) and human leukocytes from peripheral blood (Winzler see p 567 of this volume) Mannell and Rossiter (15) found that most tissues were able to label RNA purines from formate $C^{14}$ The rabbit exudate polymorphonuclear leukocyte would thus appear to be unusual

The labeling of the RNA of rabbit leukocytes from inorganic $P^{32}$ was to be anticipated from the results of early *in vivo* studies (8) and *in vitro* studies with rat spleen (10) and lymphocytic cells from rabbit appendix and mouse spleen (13)

Figures have not been given for deoxyribonucleic acid (DNA) With formate $C^{14}$ the purines of the liver DNA were labeled but not those of the leukocyte DNA With inorganic $P^{32}$ the labeling of RNA was much greater than that of the DNA in both the liver slices and the leukocytes Indeed because of the possibility that nonradioactive DNA may be contaminated with slight traces of much more radioactive RNA it is by no means certain whether the DNA is labeled at all in *in vitro* studies such as those reported above with polymorphonuclear leukocytes and liver slices and those referred to previously with brain slices (7) spleen (10) or lymphocytic cells (13) That the DNA is not labeled from inorganic $P^{32}$ would be in conformity with the suggestion that inorganic $P^{32}$ is incorporated into DNA only during cell division (11 16 17 20)

## Summary

1 When rabbit peritoneal exudate polymorphonuclear leukocytes or rat liver slices were allowed to respire in a Krebs Ringer bicarbonate buffer containing inorganic $P^{32}$ radioactivity was observed in all four RNA nucleotides The specific activities of the ribonucleotides of the leukocytes were greater than the specific activities of the corresponding ribonucleotides of the liver slices

2 When formate $C^{14}$ instead of inorganic $P^{32}$ was the source of the radioactivity labeling was observed in RNA adenine and guanine of the liver slices but not in the corresponding purines of the RNA of the polymorphonuclear leukocytes

## REFERENCES

1 Abrams R and Goldinger J M *Arch Biochem* **35** 243 (1952)
2 Brown D M Fasman G B Magrath D I Todd A R Cochran W and Woolfson M M *Nature* **172** 1184 (1953)
3 Carter C E and Cohn W E *Federation Proc* **8** 190 (1949)
4 Caspersson T *Skand Arch Physiol* **73** Suppl 8 (1936)
5 Davidson J N and Smellie R M S *Biochem J* **52** 594 (1952)
6 Davidson J N and Smellie R M S *Biochem J* **52** 599 (1952)
7 DeLuca H A Rossiter R J and Strickland K P *Biochem J* **55** 193 (1953)
8 Erf L A and Lawrence J H *Ann Internal Med* **15** 276 (1941)
9 de Haan J *Arch neerl physiol* **2** 674 (1918)
10 Heidelberger C and Keller R A *Cancer Research* **15** Suppl 3 106 (1955)
11 Hull W and Kirk P L *J Gen Physiol* **33** 335 (1950)
12 Klym J N and Cohn W E *J Am Chem Soc* **76** 1818 (1954)
13 Kit S Bacila M and Barron E S G *Biochim et Biophys Acta* **13** 516 (1954)
14 Logan J E Heagy F C and Rossiter R J *Endocrinology* **56** 455 (1955)
15 Mannell W A and Rossiter R J *Biochem J* **61** 418 (1955)
16 Marshak A *J Gen Physiol* **25** 275 (1941)
17 Osgood E E Li J G Tivey H Duerst M L and Seaman A J *Science* **114** 95 (1951)
18 Schmidt G and Thannhauser S J *J Biol Chem* **161** 83 (1945)
19 Skipper H E *Cancer Research* **13** 545 (1953)
20 Stevens C E Daoust R and Leblond C P *J Biol Chem* **202** 177 (1953)
21 Thorell B *Acta Med Scand* **129** Suppl 200 1 (1947)
22 Totter J R *J Am Chem Soc* **76** 2196 (1954)
23 Volkin E and Carter C E *J Am Chem Soc* **73** 1519 (1951)
24 Williams A D Slater G G and Winzler R J *Cancer Research* **15** 532 (1955)
25 Wyatt G R *Biochem J* **48** 584 (1951)

# General Discussion

DR A S WEISBERGER (Cleveland Ohio) I should like to direct my question to Dr Valentine and ask him to discuss something that he passed over rather briefly in his presentation namely the reciprocal relationship between sulfhydryl compounds such as cysteine and glutathione and the activity of alkaline phosphatase He found that there was a decreased alkaline phosphatase in chronic myeloid leukemia and it has been frequently reported that the sulfhydryl content of these leukocytes is elevated I should like to know if Dr Valentine believes that the high sulfhydryl content of these leukocytes may be related to the low alkaline phosphatase activity

The other thing he noted was that certain substances such as x ray cortisone and Myleran produced an elevation in alkaline phosphatase All these things have been reported to inactivate sulfhydryl compounds Thus the mechanism of decreasing the inhibitor as he mentioned might be operating here

DR WILLIAM C MOLONEY (Boston Massachusetts) Our efforts have been directed much along the lines of Dr Valentine s however we have used histochemical as well as biochemical methods to study the behavior of alkaline phosphatase activity of polymorphonuclear leukocytes

Our data support his observations that there are probably two polymorphonuclear leukocyte populations in chronic myelogenous leukemia This can be demonstrated by the fact that during the course of a pyogenic infection in patients with chronic myelogenous leukemia there is a moderate rise in alkaline phosphatase activity shown by biochemical methods With histochemical techniques however this increased phosphatase activity is confined to about 20% of the segmented neutrophils In contrast in normal individuals during pyogenic infection over 90% of the segmented neutrophils become strongly positive

Prolonged incubation will produce a similar phenomenon and such observations indicate methods of investigating this cell population problem

In passing I should also like to mention that we have studied the effect of Myleran x ray and $P^{32}$ in myeloid metaplasia and in leukemic populations We have noted that there has been no shift in alkaline phosphatase activity toward normal values This may mean that these agents uniformly affect the blast cell before differentiation in both hyperplastic and leukemic populations

We have studied 5 nucleotidase activity in polymorphonuclear leukocytes and have found that cells with nonspecific alkaline phosphatase split the phosphate from the 5 carbon of adenosine 5 phosphate as a nonspecific phosphomonoesterase

DR RICHARD J WINZLER (Chicago Illinois) Dr Valentine has raised the very interesting possibility that the remaining cells of the leukemic patient in remission may not be normal but may represent a lineage derived from the abnormal leukemic cells this being reflected in a low alkaline phosphatase activity

We have some data bearing on this point Leukocytes from patients with granulocytic leukemia take up radioactive formate more rapidly than normal or lymphatic leukemia cells and are much more sensitive *in vitro* to folic acid antagonists In several such patients treated with Myleran we have found that as the cell count returns to normal the metabolism of the cells as reflected by formate uptake and sensitivity to amethopterin also returns toward normal This would lead to a con

clusion opposite from that Dr Valentine derived from his work with alkaline phosphatase

I think this question of the normalcy of leukocytes during a leukemic remission is very much a key point that we should keep in mind in future research

Dr George Rouser (Duarte California) Dr Valentine how does variation in cell size affect the per cell phosphatase value?

Another question I should like to direct to Dr Rossiter Are the rabbit exudate cells permeable to formate? If they are as seems likely it may be that the failure to observe formate incorporation into cell nucleic acid in contrast to ready $P^{32}$ incorporation is due to the absence of free glutamine in the cells as has been indicated by our chromatographic results

Dr Milton Toporek (Ann Arbor Michigan) I should like to add some observations we have made on acid phosphatase in white cells isolated from leukemic cases

As shown by Swendseid the enzyme activity in the granulocytes is roughly ten times that found in the lymphocytes We followed a fair number of patients through many months and many changes in treatment in an attempt to correlate changes in acid phosphatase activity with cell populations

As has occurred so many times in such situations we came away without a definite conclusion merely a frustrating impression that some of the changes we have found were correlated with increases and decreases in the immature cell population

Dr Nathaniel B Kurnick (Long Beach California) Dr Valentine has made the interesting observation that patients with lupus erythematosus have normal or low leukocyte alkaline phosphatase activities despite the inflammatory reaction in this disease I presume many of these patients were on steroid therapy

I am wondering if Dr Valentine would comment on whether the leukocyte in lupus erythematosus is abnormal as it is in leukemia in its response to steroids or whether prolonged steroid therapy results in depression of the alkaline phosphatase in normal individuals also

Dr L D Hamilton (New York New York) I should like to ask Dr Valentine what he thinks the function of this enzyme is that he has been measuring in the white cells and what significance if any he would place on the fact that it is lower in chronic myeloid leukemia than in any other normal granulocyte

Dr Sven Moeschlin (Solothurn Switzerland) I should like to mention one point There are also clinical subfindings which point to the possibility of two populations in leukemia

I think there is no doubt in some cases of acute leukemia (Dr Bernard in Paris and I have seen this in puncturing different marrow cells in the very early cases of leukemia) that there were completely different findings Central myeloblasts were in the sternal marrow and not in others I think we will find in the end that with all the marrows involved it may just be denervation as we also see in some myelomas

There also are very different findings in making splenic punctures and marrow punctures Some cases show a very mature cell and blast forms in the spleen and others in the marrow and so on So there may be in some cases of chronic myeloid leukemia a difference in those two populations

# General Discussion

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# 29

## Lactic Acid Production by Polymorphonuclear Leukocytes[1]

SAMUEL P MARTIN GORDON R McKINNEY[3]
R GREEN and JANE DERBYSHIRE

Department of Medicine Duke University School of Medicine Durham North Carolina

The mature polymorphonuclear leukocytes from normal subjects possess a high aerobic lactic acid formation (4) These cells do not exhibit the "Pasteur effect" in that the lactate formation is not increased by exclusion of oxygen from the environment (10) The retina (23) certain nervous tissues (23) renal medulla (6) and intestinal mucosa (7) share this characteristic with the leukocyte In contrast myeloid leukemic cells have a low lactic acid formation in the presence of oxygen and exhibit the Pasteur effect (10) There has been considerable study directed toward the quantitative aspects of this high glycolytic rate but few data are available on the route of glycolysis or on the possible sources of this lactic acid

One type of study that has been used to elucidate these differences in various tissues is the preparation of crystalline enzymes Lactic dehydrogenase from muscle and tumor cells was shown to have no qualitative difference (11) Various other enzymes have been studied In tumor tissue zymohexase was found to be present in limited quantity and was thought to be working at near capacity (30) In the leukocyte

[1] This work was supported in part by a research grant (No C-3476) from the National Institutes of Health United States Public Health Service the Atomic Energy Commission (Contract AT(40 1)1081) the Baxter Laboratories The American Medical Association and the American Cancer Society

Present address Department of Medicine College of Medicine University of Florida Gainesville Florida

[3] Present address Department of Pharmacology West Virginia University School of Medicine Morgantown West Virginia Lederle Medical Faculty Award Recipient

DR W N VALENTINE (Los Angeles California) First of all in answer to Dr Weisberger's questions in regard to the sulfhydryl content we measured sulfhydryl content of the leukocytes by the nitroprusside method and unlike the iodometric *method* or *the* method which Dr Martin has used we find that the glutathione content seems not to be elevated in leukemia

Dr Martin may have some comments on this in that ergothioneine and other sulfhydryl compounds of leukocytes measured by some methods and not by others is not measured by our method which measures more closely glutathione

I do not think this is the inhibitor that is possibly involved here because of the fact that in order to get substantial inhibition you have to have 0 001 to 0 002 molar concentrations If you get rid of that sort of concentration in your incubation mixture you do not have significant inhibition Therefore I do not think this is a specific cysteine or glutathione type of inhibition

Second Dr Weisberger I do not believe Myleran or x ray ordinarily affects the alkaline phosphatase of the cells in remission It was only in these exceptional instances where this was observed very early in the course of the disease and associated with a very complete type of remission In other instances the vast majority there was no change in the pattern despite much greater amounts of x ray and Myleran administered

Dr Winzler's comments were very interesting I should like to say that I put out this comment about the two-population theory not because of any brief that I have felt for it One can interpret the results just as easily as a metabolic reversion rather than on a two population theory but I think his point of differentiating between the two possibilities is of some importance

In regard to Dr Rouser's question about comparison of cell weight and its possible effect on enzymes we find that on a cell nitrogen basis in chronic myelocytic leukemia the unit cell nitrogen content is very nearly that of the average cell in the normal population It runs about 0 9 of normal and is a little less in terms of nitrogen content In chronic lymphatic leukemia which we are not dealing with here it is appreciably less running about 40 or 45% in our hands

In regard to the acid phosphatase situation we have measured that in cells but we do not find this striking lability in acid phosphatase that we find in alkaline phosphatase nor do we find it reduced in leukemia

Concerning Dr Kurnick's question the alkaline phosphatase in the subjects with lupus erythematosus was not low but was within the normal range What this means I don't know Some of these patients had been on large doses of steroid for considerable periods of time and the values were still low

Whether this represents an abnormal leukocyte or whether in some of these collagen diseases (and I believe there is ancillary evidence for this in other areas) the so-called "stress or the obvious fact that the patient is sick does not necessarily evoke an increased pituitary adrenal response I don't know but I suspect that at least in some cases while the patient is ill and is under "stress in certain types of diseases this does not necessarily evoke the response

For instance in rheumatoid arthritis the value may be normal in the presence of elevated sedimentation rates and other evidences of activity nonetheless if you give these patients ACTH they get the rather characteristic normal elevation

# 29

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of the horse Wagner has shown enzymatic degradation of glycogen however there were no reducing intermediates between glycogen and lactic acid in the intact cell (26) In dialyzed cells reducing intermediates were found to be present and the lactic acid formation was proportional to the phosphate concentration (27 28) The intermediates isolated were shown to be glucose 1 phosphate glucose 6 phosphate fructose 6 phosphate fructose 1 6 diphosphate and phosphoglyceric acid He also showed the presence of hexokinase (29)

Recently Beck carried out an excellent study on the kinetics of glycolysis and certain glycolytic enzymes in the normal mature and leukemic leukocytes (3) These studies add enormously to the knowledge of the glycolytic system in normal and leukemic cells He showed that these cells contain all the enzymes of the Meyerhof Emden scheme of glycolysis (Fig 1 steps 5 through 12) In the quantitative measurement of enzyme concentration the leukemic cell contained greater aldolase and isomerase activity (Fig 1 steps 5 and 6) but less glyceraldehyde-3 phosphate dehydrogenase and lactic dehydrogenase (Fig 1 steps 7 and 12) The latter enzymes were proportional to the lower aerobic lactic acid formation of the leukemic cell He also showed that despite the proportionality between dehydrogenases and glycolytic activity the capacity far exceeded actual production The aldolase activity of these cells like that of tumor cells seemed to be working at near capacity Purified lactic dehydrogenase from the two cell types was shown to have identical behavior and no differences were demonstrated with regards to Michaelis constants or pH activity relationships of the other enzymes These activities agreed with those of enzymes from other sources No rate limiting enzyme step was found The enzymes involved in the conversion of glucose to fructose 1 6 diphosphate were not evaluated in this report Broken cells were used and thus there was no evaluation of the characteristics of the cell membrane The rate limiting action may be found in this area of glucose transport or phosphorylation since the studies of Wagner using intact cells showed no accumulation of reducing intermediates

Recent studies have indicated that the glucose oxidation and the hexose shunt (Fig 1 steps 13 through 16) may play an active role in the glucose utilization of tissues such as the adrenal gland and certain lymphomas (8) The importance of this step in the metabolism of the polymorphonuclear leukocyte and its possible role in the high aerobic glycolytic rate await further study

The intact mature leukocyte shows an increase in lactic acid production on the addition of glucose This increase is proportional to the con

centration of glucose until levels of 100 mg % are reached (19) (Fig 2) The response to fructose differs from that of glucose in that the lactic acid production is directly *proportional to the concentration of fructose* over a wide range. On the addition of combinations of glucose and fruc

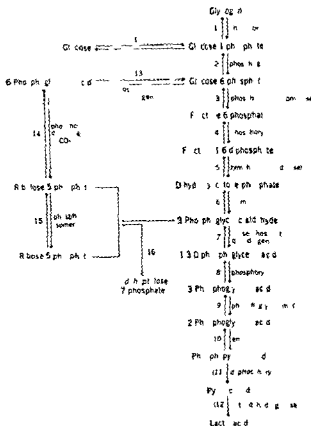



FIG 1 The Meyerhof Embden System of Glycolysis and the Hexose Monophosphate Shunt

tose the lactic acid production is increased however this increase comes about through the increased utilization of glucose

Studies have been carried out in this laboratory on the effect of inhibitors of glycolysis on the lactic acid production of the mature human granulocyte in an effort to determine some of the characteristics of this cell A review of these has previously been published (16 19) The most striking effect is a negative one and is presented by oxamic acid in

hibitor of lactic acid dehydrogenase (9) This compound has a slight effect on the lactic acid production however this could be overcome by the addition of pyruvate Iodoacetate was the most potent inhibitor If the cells were exposed to iodoacetate and then dialyzed lactic acid could still be produced in the presence of hexose diphosphate This would *tend to indicate that another route might exist for lactate production* which does not involve the enzyme glyceraldehyde-3 phosphate de

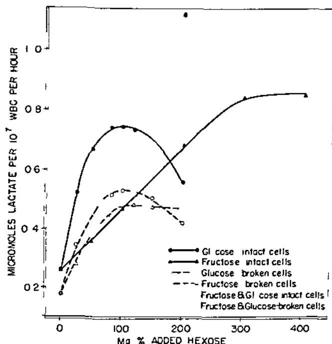



Fig 2 The effect of glucose and fructose on the aerobic production of lactic acid by leukocytes from healthy subjects

hydrogenase since this enzyme has as its prosthetic group glutathione (5)

*Various substrates have been studied with the mature human leuko* cyte The intact cell cannot utilize the phosphorylated intermediates It is interesting that in the broken unfortified cell system more lactic acid is produced from glyceraldehyde-3 phosphate and pyruvate than from 3 phosphoglyceric acid

The role of various cofactors in lactic acid production is under study at the present time In dialyzed cells glutathione increased the lactate production Magnesium and phosphate *appear to be necessary for* lactic acid formation from glucose

Recent studies in this laboratory have added to the complexity of the problem of lactate production. Lactate analyses have been made on extracts of polymorphonuclear leukocytes and erythrocytes. The Barker Summerson method (1) has been compared with the modification of the method described by Neilands (22) using a purified lactic acid dehydrogenase. With erythrocytes there is close agreement between the values derived by the two techniques. But with leukocytes the Barker Summerson method gives values consistently greater. This would indicate the formation of some compound other than L lactic acid.

Stereochemical studies in the past indicate that lactic acid produced by muscle is L lactic acid. Levene showed that leukocyte of pleural exudate of dogs in the presence of glucose produced L-lactic acid and with methylglyoxal DL and L (12 14). Racker (24) stated that glyoxylase I from yeast has a specificity for the production of D-lactic acid from methylglyoxal and glyoxylase II from muscle can produce either D or L lactic acid depending on the intermediate used.

The role of methylglyoxal as an intermediate in carbohydrate metabolism has been a stormy one and has recently been relegated to the secondary role of the removal of spontaneously formed methylglyoxal in muscle (21). McKinney and co workers have recently reviewed the possible activity of glyoxylase in the mature human leukocyte. It is interesting to note that earlier studies in which this enzyme was thought to play an important role in the glycolytic system were carried out on blood cells whereas later more definitive studies were carried out on muscle (2 25 31 32). Barrenscheen (2) isolated the semicarbazine of methylglyoxal from dogs blood. Schneider and Widmann (25 31 32) demonstrated that human leukocytes form methylglyoxal and stated that 70 to 75% of the glucose passed through this stage. Preliminary observations in our laboratory indicate the formation of methylglyoxal by dialyzed human granulocytes. McKinney (15 16) has shown that the mature granulocyte contains more than 700 times the glyoxylase activity of the erythrocyte. In addition he has shown that the glyoxylase activity of the mature polymorphonuclear leukocyte is three times that of the leukemic cell (17). This latter quantitative difference might account for some of the aerobic lactic acid production and the absence of the Pasteur effect in the mature leukocyte since he has shown that the glyoxylase of leukocytes is not influenced by anaerobiosis nor is it inhibited by oxamic acid.

The lactic acid production can be shown to be influenced by disease states as well as hormone concentration (18). Adrenal steroid hormones compounds E and F in physiologic concentration cause a decrease in

lactic acid production The leukocytes from diabetic individuals have a low lactic acid production and this is increased on the addition of insulin (20) This response to insulin is not seen in the normal leukocyte Leukocytes respond to the presence of bacterial pyrogens with increased lactic acid production This makes the use of pyrogen free water important in physiologic studies (19)

The studies thus far do not give a definitive answer to the factors underlying the high aerobic lactate production of the mature polymorphonuclear leukocyte One must await further studies such as those of Beck involving enzymes which transport glucose across the cell membrane and other enzymes involved in the production of fructose 1 6 diphosphate The role of the hexose shunt as a possible source of lactic acid production also awaits further study The place of glyoxylase must also await evaluation

The present studies however indicate that this cell is very sensitive to many changes in the internal environment It is interesting to speculate on its future use as a laboratory tool for the study of cellular mechanisms of disease Studies such as those of Valentine Kieler Hamilton Vilter and Weisberger reported at this Symposium make this speculation a rather safe one It is a cell that can be easily obtained in the free state It responds rapidly to environmental changes such as hormone concentration And the most important characteristic of this cell is that it can be removed from the body thus removing it from the well adjusted compensatory mechanisms which have limited our studies in the past where body fluids have been our only source of material

## REFERENCES


1 Barker S B and Summerson W H *J Biol Chem* **138** 535 (1941)
2 Barrenscheen H K *Biochem Z* **193** 105 (1928)
3 Beck W S *J Biol Chem* **216** 333 (1955)
4 Beck W S and Valentine W N *Cancer Research* **13** 309 (1953)
5 Cori G T Stein M W and Cori C R *J Biol Chem* **173** 605 (1948)
6 Dickens F and Weil Malherbe H *Biochem J* **32** 567 (1938)
7 Dickens F and Weil Malherbe H *Biochem J* **35** 7 (1941)
8 Glock G E and McLean P *Biochem J* **66** 171 (1954)
9 Hakala M Glaid A J and Schwert G W *Federation Proc* **138** 535 (1953)
10 Kempner W *J Clin Invest* **18** 291 (1939)
11 Kubowitz F and Ott P *Naturwissenschaften* **29** 509 (1941)
12 Levene P A and Meyer G M *J Biol Chem* **11** 361 (1912)
13 Levene P A and Meyer G M *J Biol Chem* **14** 149 (1913)
14 Levene P A and Meyer G M *J Biol Chem* **14** 551 (1913)
15 McKinney G R *Arch Biochem and Biophys* **46** 246 (1953)
16 McKinney G R and Martin S P *Blood* in press
17 McKinney G R and Rundles R W *Cancer Research* in press

18 Martin S P Chaudhuri S N Green R and McKinney G R *J Clin Invest* 33 358 (1954)
19 Martin S P McKinney G R and Green R *Ann N Y Acad Sci* 59 996 (1955)
20 Martin S P McKinney G R Green R and Becker C *J Clin Invest* 32 117 (1953)
21 Meyerhof O *Experientia* 4 169 (1948)
22 Neilands J B in "Methods of Enzymology" (S P Colowick and N O Kaplan eds) Vol I p 449 Academic Press New York 1955
23 Quastel J H *Physiol Revs* 19 135 (1939)
24 Racker E in Glutathione (S Colowick A Lazarow E Racker D R Schwarz E Stadtman and H Waelsch eds) p 208 Academic Press New York 1954
25 Schneider E and Widmann E *Klin Wochschr* 9 1910 (1930)
26 Wagner R *Arch Biochem* 26 123 (1950)
27 Wagner R and Reinstein S E *Arch Biochem* 29 260 (1950)
28 Wagner R and Yourke A *Arch Biochem and Biophys* 39 174 (1952)
29 Wagner R and Yourke A *Arch Biochem and Biophys* 44 415 (1953)
30 Warburg O and Christian W *Biochem* Z 314 509 (1943)
31 Widmann E *Biochem* Z 216 479 (1929)
32 Widmann E and Schneider E *Biochem* Z 224 157 (1930)

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7 Dickens F and Weil Malherbe H *Biochem J* **35** 7 (1941)
8 Clock G E and McLean P *Biochem J* **56** 171 (1954)
9 Hakala M Glaid A J and Schwert, G W *Federation Proc* **138** 535 (1953)
10 Kempner W *J Clin Invest* **18** 291 (1939)
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12 Levene P A and Meyer G M *J Biol Chem* **11** 361 (1912)
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# 30

# Diagnostic and Nosological Problems at the Borderline of Leukemia

F G J HAYHOE

Department of Medicine Haematological Clinic,
University of Cambridge Cambridge England

The histologist seeking to ascertain the nature of a pathological lesion looks with particular care at the periphery where the transition from abnormal to normal tissue takes place The features to be seen at this interface often prove of diagnostic importance particularly in neoplastic and necrotizing processes There is some justification for adopting a similar approach to the interpretation of disease states and supplementing the information obtained from the study of classical manifestations of a disorder by particularly careful attention to incomplete or atypical forms

There is no scarcity of such borderline states in the case of leukemia Many of them are remarkably interesting and must be closely considered when the fundamental nature of the leukemic disorder is discussed I propose to make some brief comments supported by occasional reference to case data about several of the most clearly recognized of these "paraleukemic" states and attempt to illustrate and expand the idea that leukemia may be merely one manifestation of an abnormal process capable of showing itself in several different clinical and hematological forms

## The Relationship of Aplasia to Leukemia

Three particular aspects of this relationship may be delineated

1 Transition may occur from an apparently typical pancytopenia with marrow aplasia and no increase in primitive cells to a fully leukemic picture The salient features of one such case are shown in Fig 1 The change from a pancytopenic aplastic nonleukemic condition at the onset to a normal picture and thence to a frankly leukemic one is clearly

2 Aplastic phases may develop during the course of leukemias either treated or untreated and such a change is by no means confined to cases where vigorous use of antimitotic agents can be held responsible Spontaneous remissions may be heralded by an agranulocytic hypoplastic or aplastic marrow picture as so well exemplified in the striking case reported by Bassen and Kohn (2) where four remissions unassociated with specific antileukemic treatment apart from transfusions were each preceded by marrow hypoplasia

3 Pancytopenias which never become frankly leukemic frequently show some increase in primitive cells in the marrow and as many as 20% of the marrow cells may be blast forms The development of maturation arrest at the myeloblast promyelocyte stage in response to granulocytopenogenic agents has been clearly demonstrated by Moeschlin and his associates (16) and in such experiments although the peripheral blood picture is one of agranulocytosis the marrow may become very *like that of leukemia*

Example of each of these three kinds of aplasia leukemia relationship are sufficiently common to influence our thinking on the etiology and pathogenesis of leukemia and the correlation is further supplemented by the actions of agents such as benzene and x rays which may produce either leukemic or aplastic pictures Explanations in terms of irritative hyperplasia and "exhaustion atrophy are unsatisfactory unless we can say what exactly is being irritated and what exhausted

### Leukemia Myeloproliferative States and Aplasia

The relation of leukemia and aplasia may be further expanded if we consider leukemia as one of a group of myeloproliferative disorders in which are included myelofibrosis megakaryocytic myelosis polycythemia vera and erythremic myelosis This concept adumbrated by many authorities in the past has been clearly expounded and amply illustrated in recent years (5 10 11) and has probably been fairly generally accepted

The similarities between members of this group and the transitions which may occur between them are now well recognized and I do not propose to spend much time amplifying this point but Figs 2 through 5 illustrate one or two of the most important transitions and intermediate states

Since aplastic pancytopenic phases may occur either terminally or during the course of any of these myeloproliferative *disorders we may* draw a chart of interrelationships of the kind shown in Fig 6

shown It is noteworthy that three marrow punctures at different sites during the early stages of this disease failed to reveal any increase in primitive cells

A similar sequence of events has been recorded in many carefully studied cases (3 12 14 20) and one may be right in suspecting that the

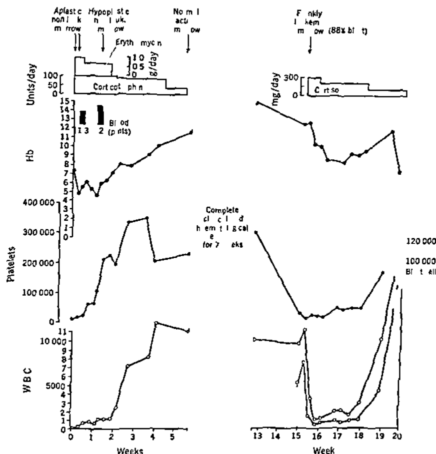



FIG 1 Transition from typical pancytopenia to a fully leukemic picture (Reproduced by courtesy of Dr Gibbs of Addenbrooke s hospital )

sequence has occurred a good deal more frequently than it has been reported In this connection the report of Stodtmeister and Buchmann (18) that small islands of leukemic cells were often to be found at thorough post mortem examination in cases of aplastic anemia is most suggestive of a very close relation between idiopathic aplasia and acute leukemia

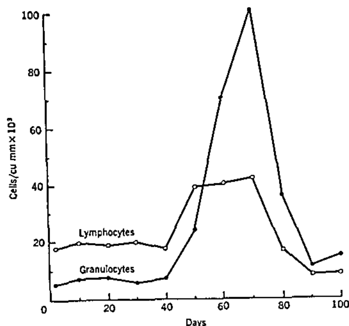



Fig 4 Graph depicting the peripheral blood changes in a case of myelofibrosis during a particularly interesting phase of the disease Remarkable fluctuations of lymphocyte and granulocyte levels gave successive resemblances to chronic lymphatic and chronic myeloid leukemia

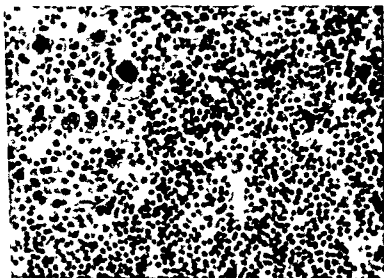


Fig 5 Marrow section from the patient whose blood changes were shown in Fig 4 It shows increased connective tissue megakaryocytic hyperplasia and lymphocytic infiltration

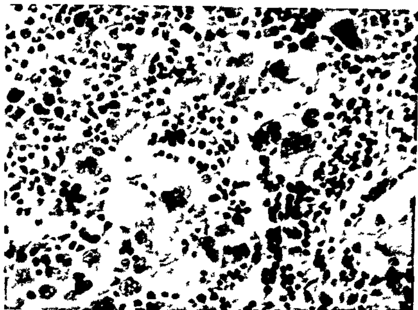


FIG 2 Marrow section from a case of megakaryocytic myelosis showing a proliferative hyperplastic picture involving all marrow cell series but particularly the megakaryocytes There was some general increase in connective tissue and reticulin The peripheral blood of this patient showed a mild leukoerythroblastic (myelophthisic) anemia

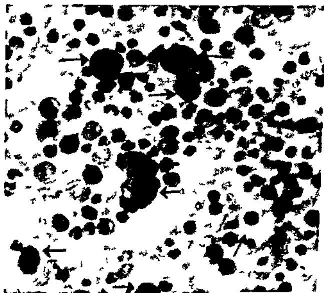


FIG 3 Photograph of a marrow smear from a patient with chronic myeloid leukemia having a typical peripheral blood picture Gross megakaryocytic hypertrophy is evident with many megakaryocyte precursors

atypical tuberculosis If we accept the tuberculous cases as having a single cause and common pathogenetic mechanism we cannot escape the strong suspicion that a comparable state of affairs exists in the parallel idiopathic" group If this is so biochemical and virus studies designed to detect a fundamental metabolic defect in leukemia ought to be directed equally to the detection of a similar defect in aplastic cytopenic and myeloproliferative states The biochemical and virological similarities rather than differences would be of major significance

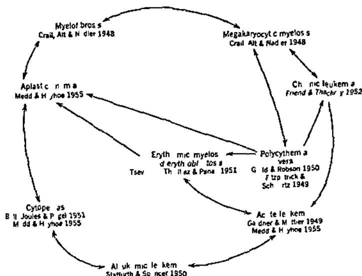



FIG 7 Figure 6 supplemented by references to case reports of tuberculous parallels to each of the disorders included in the figure

Although discussion of the leukemic remission is not strictly germane to the present argument I feel compelled to make some brief concluding reference to it since it is in a sense a very remarkable paraleukemic state

Remissions occurring spontaneously or after transfusion or treatment with steroid hormones or antimitotic agents frequently satisfy the criteria laid down by most investigators that both marrow and peripheral blood should return substantially to normal and that physical signs and symptoms of leukemia should disappear These striking remissions lasting from a few weeks to more than a year must influence our conception of the basic leukemic process which is clearly of a kind susceptible to apparently complete temporary reversal I should however like to pose a

## Myeloproliferative and Pancytopenic Reactions to Tuberculosis

With this diagram of relationships (Fig 6) in mind I should like to draw attention to the remarkably similar series of hematological abnormalities which may be encountered during the course of atypical tuberculosis Dr Medd and I *in a recent article on tuberculous miliary* necrosis with pancytopenia (15) discuss some of the reported examples of these peculiar blood reactions and point out that they provide a striking parallel to the primary myeloproliferative and pancytopenic group of disorders I think it is true to say that every one of the conditions included in Fig 6 has been reported to be mimicked by a reaction to tuberculosis In support of this statement I have drawn a second version of Fig 6 (Fig 7) in which at least one reference to recorded cases of the tuberculous parallel is given for each of the disorders mentioned

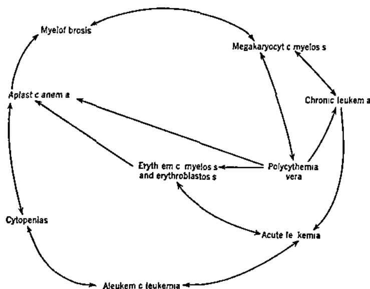



FIG 6 Interrelationships of myeloproliferative and aplastic states

## Speculations on the Background and Nature of Leukemia

The general conclusion that can be drawn from this survey of paraleukemic states is that the leukemias form only one aspect of a wide group of related diseases including not only the myeloproliferative disorders but also cytopenic and aplastic ones The close relationship of this large group of diseases is further emphasized by the occurrence of a similar pattern of blood and marrow pictures during the course of

As a clinician I always defer to the pathologist since the diagnosis of leukemia is a pathologist's diagnosis first, last and always At best the clinician's diagnosis of leukemia is an inferential one

The evidence that is indicated in the literature remarked on by Dr Moeschlin Dr Doan Dr Dameshek and refreshed in our memories this morning by Dr Hayhoe indicates that these queer transitions occur not only in the clinical manifestations of these diseases but even in their cytology facts which were magnificently brought out by Hal Downey many years ago The question is who has the temerity to make the diagnosis?

The next thing is this I have watched the pathologist at the autopsy table and during dissection and finally after the microscopic examination of tissues of a case of treated leukemia say I cannot make a diagnosis of leukemia in this case If it had existed certainly what you did has blasted it out of recognition There is no evidence diagnostically of leukemia"

The question is were we dealing with one of these queer cantrips or one of these caprices of this very primitive magnificent and marvelous tissue the study of which has captured our attention in its overtones and fantasies these past three days?

Dr Sergio De Carvalho (Cleveland Ohio) I should like to ask a question concerning Dr Hayhoe's paper and then express a wish Do you think that you can compare from the cytological point of view the terminal myeloblastic reaction of a chronic myeloid leukemia with an acute leukemia d emblee? I don't

I wish you would develop the relationship between the megaloblastic state and the initiation of acute leukemia a relationship we observed lately in four cases

Dr W N Valentine (Los Angeles California) I should like to raise one point in connection with Dr Hayhoe's paper It seems to me that in dealing with a tissue which is multipotential we are still limited to a certain number of responses which can occur to a variety of stimuli This particular type of tissue presumably can differentiate into different types of blood cells It can differentiate into fibrous tissue from the reticulum and into bony tissue It does not appear to me to be utterly convincing that because reactions resemble each other superficially and because they have morphologic similarities they are necessarily related pathogenetically in view of the fact that the scope of the reactions is also limited to some extent

Dr Albert S Gordon (New York New York) I should like to draw some parallels between the observations of Dr Martin and certain of the experiments that we have conducted relating to the matter of glycolysis within the blood forming organs

Dr Martin has confined his remarks to the mature human polymorphonuclear leukocyte whereas our studies have centered largely on blood forming organs with particular emphasis on the bone marrow of the rat

Quite interestingly after removal of the adrenals in rat there is an increase in the glycolytic activity of the bone marrow This metabolic change is accompanied by an increase in the relative immaturity of the neutrophilic granulocytic population an increase in the absolute numbers of lymphocytes and eosinophils and a decrease in the numbers of nucleated red blood cells

Of course we don't know here what is cause and effect It may be that the metabolic changes which must necessarily precede the morphologic ones in differen

question in relation to the extent of these complete remissions Do all pathological manifestations of the disease in fact ever disappear? Certainly in a small proportion of cases no cytological abnormality whatever can be detected either by ordinary staining methods or by histochemistry If there is no detectable evidence of leukemia during such a remission why should relapse be invariable?

## REFERENCES

1 Ball K Joules H and Pagel W *Brit Med J* ii 869 (1951)
2 Bassen F A and Kohn J L *Blood* 7 37 (1952)
3 Block M Jacobson L O and Bethard W F *J Am Med Assoc* 152 1018 (1953)
4 Crail H W Alt H L and Nadler W H *Blood* 3 1426 (1948)
5 Dameshek W *Blood* 6 372 (1951)
6 Fitzpatrick W J and Schwartz S O *Am Rev Tuberc* 60 660 (1949)
7 Friend J and Thackray A C *Brit Med J* i 574 (1952)
8 Gardner F H and Mettier S R *Blood* 4 767 (1949)
9 Guild A A and Robson H N *Edinburgh Med J* 57 145 (1950)
10 Heller E L Lewisohn M C and Palin W E *Am J Pathol* 23 327 (1947)
11 Hutt M S R Pinniger J L and Wetherley Mein G *Blood* 8 295 (1953)
12 Mallarme J *Le Sang* 20 429 (1949)
13 Marchal G Deprez V and Blanc G *Le Sang* 16 133 (1944)
14 Meacham G C and Weisberger A S *J Lab Clin Med* 42 923 (1953)
15 Medd W E and Hayhoe F G J *Quart J Med* 24 351 (1955)
16 Moeschlin S Meyer H Israels L G and Tarr Gloor E *Acta Haematol* 11 73 (1954)
17 Staffurth J S and Spencer H *Blood* 5 1161 (1950)
18 Stodtmeister R and Buchmann P *Ergebn inn Med u Kinderheilk* 60 367 (1941)
19 Tsevrenis H Thuilliez A C and Ianas G *Le Sang* 22 78 (1951)
20 Williams M J *Blood* 10 502 (1955)

## General Discussion

Dr Salvatore Lucia (San Francisco California) I have no restraints in remarking on Dr Hayhoe s paper especially since many of the things I have heard these last three days are so definitive that I begin to doubt my very existence in an atmosphere of science limited to conditions which apparently have offered no challenge to the diagnostician

What I have to say now is not too comforting to those of us who are engaged in definitive research and those of us who are challenged with the responsibility of making a diagnosis of the presence or not of leukemia

cytes with a paucity of platelets and with rapid destruction of erythrocytes The initiating, sustaining, and compensatory mechanisms that are involved now demand that the concept of the leukemias in the future must be on a broader basis and that we cannot focus on one specific el ment as we have done today

Dr Wayne Rundles (Durham North Carolina) The importance of reduced bone marrow function or cytopenias in myelogenous leukemia, I think, has been emphasized with complete justification This type of patient seems to be in creasingly common and may be significant in reference to factors producing leukemia

In reference to other types of malignant proliferations of bone marrow we have had two individuals with rather typical chronic granulocytic leukemia as judged by the immature circulating granulocytes and associated changes in the bone marrow who in addition have had plasma cell proliferations that we would regard as diagnostic of multiple myeloma These individuals have had not only numerous abnormal plasma cells in their bone marrow but homogeneous globulin components in their sera

In reference to "pseudoleukemia" induced by infection some patients with well documented myelogenous leukemia eventually are found at autopsy to have active tuberculosis fungus diseases and even such bizarre entities as inclusion cell disease" When little is known about a given patient s hematologic background, there is always a question as to whether these developments have come into the picture at a late hour or whether they actually do simulate or produce a leukemic blood picture

Chairman C A Doan (Columbus Ohio) We have seen in our clinic a propos of Dr Hayhoe s comments a number of patients with hypoplastic marrow and a pancytopenia as the initial hematologic picture and after many weeks or months a terminal blastic leukemia has developed It has been our definite impression that these cases have terminated as monoblastic not myeloblastic leukemia When the marrow is examined supravitally the basic mesenchymal reticuloendothelium only is seen giving rise to primitive monoblasts somewhat less differentiated than the myeloid cell series The question is whether Dr Hayhoe has also found the terminal phase in these original hypoplastic states to represent a monoblastic rather than a myeloblastic differentiation

Dr F G J Hayhoe (Cambridge England) I certainly have bitten off a great deal more here than I can chew Seven problems have been presented almost any one of which would take twenty minutes to discuss I will run through them very rapidly

First Do you think that the myeloblastic termination of chronic myeloid leukemia is the same as acute leukemia?" Your guess is as good as mine Cytologically it may be precisely the same In general, however the response to treatment is dif ferent For example we had some nice illustrations of the response of this myeloblastic stage to deacetylmethylcolchicine and in my experience with acute leukemias they respond very very poorly to deacetylmethylcolchicine So it is possible on the basis of therapeutic results that there is something strikingly dif ferent but cytologically they may be very similar

Second the megaloblastic onset of leukemias I myself ha e not seen a case of leukemia developing in what had previously been a megaloblastic picture although I have often encountered megaloblasts in an already de eloped leukemic picture

tiation processes are responsible for the structural changes that occur in the marrows of the adrenalectomized animal

*Similarly* if one administers adrenal cortical steroids the increase glycolysis is overcome and the morphology is restored to normal Moreover one can depress the rate of glycolysis below normal levels by giving larger quantities of the steroid

Recently we have been considering the possibility that the changes which occur after adrenalectomy may not be mediated directly but may be a consequence of alterations in the vascular dynamics involving the affected organ Thus the blood flow through vital organs of the adrenalectomized animal may be impaired Hoagland and his associates have demonstrated a decrease in the flow of blood through the head of the adrenalectomized rat which can be restored to normal by the administration of certain adrenal steroids

Since a number of people have been going out on limbs this morning I should like to select one for myself and suggest that adrenalectomy by causing ischemia within the bone marrow sets into motion some abnormal metabolic pattern which precedes and may be responsible for the observed morphologic changes Similarly adrenal steroids may exert their ameliorative effects in the adrenalectomized animal and perhaps also in the leukemias by correcting the abnormal metabolic changes

All this might correlate with Dr Hayhoe s observations on leukemia in patients with tuberculosis or cardiovascular disease in whom some degree of anoxia may very well exist Obviously not all such subjects develop blood dyscrasias but the possibility exists that the sensitivity of the blood forming organs to hypoxia is more acute in the susceptible subject

It would seem a possibility that some forms of pathologic blood formation are related at least in part to disturbances in the mechanism concerned with the normal oxygenation and nutrition of the blood forming tissues

DR HOWARD R BIERMAN (Duarte California) I should like to sound a note of caution

First of all the leukemias are indeed a dynamic group of diseases It should be readily conceded that there may be a phase of the leukemias certainly at their very onset in which they are neither clinically nor hematologically detectable and yet there is no doubt that these children or patients do have leukemias As Block and Jacobson showed in some of the early preleukemic cases the classical hematologic characteristics are often absent

Since we apparently have focused our attention today on the leukocytes it is worth while to emphasize a bit that these diseases do not restrict themselves exclusively to the leukocytes The leukemias involve all the hematological elements The leukocytes the erythrocytes and the platelets are equally involved and yet it also must be realized that perhaps none of these elements may contain the fundamental defect of these diseases

The key biochemical lesion of the leukemias has not been revealed at present If thus is a disease of overproduction of the leukocytes as Virchow originally said then x ray therapy I$^{3}$ arsenic nitrogen mustard the antimetabolics and all the other agents which are very effective leukocyte destroyers would have produced a cure in at least a few instances

Therefore it might be wise to keep an open mind and entertain the possibility that the present basic concept of the leukemic state is incorrect We are dealing with a group of fatal diseases characterized by the accumulation of immature leuko-

## Part VIII

# Nucleic Acids as the Target for Chemotherapy Mechanisms of Drug Action and Resistance

Chairman
**Joseph H Burchenal**
Memorial Center for Cancer and Allied Diseases
New York New York

Without chasing down the literature on this subject unless there is some more informed person who can tell us I wouldn't like *to say anything about megaloblastic* states of onset It is not difficult to imagine that our nuclear protein chemists can easily offer a rational kind of explanation of why we might expect to find megaloblastic change in an acute leukemic state

I should like to comment on Dr Valentine's remark Of course I am not in the least suggesting that there is any conclusive evidence of an etiological or pathogenetic identity between all these states I am merely putting up a scheme to stimulate ideas Certainly I think there is a relationship between these conditions and we ought not ignore it

The next question concerns the importance of anoxia in pathogenesis I think this is an extraordinarily interesting topic but it is one into which I don't think we can really go

During the last week I had the opportunity to read Warburg's article in *Science* which *I think is very relevant to this point I don't think I would add anything other* than that I find Warburg's arguments on the matter a very fruitful sort of stimulating presentation with regard to the importance of anoxia in the development of later productive states

The next question asked was on the importance of a broad approach to the matter I believe Dr Bierman asked it I absolutely agree I think it is most important that since so many of us are working in tiny isolated fields at least some of us should attempt to maintain a broad clinical approach and offer some kind of counterbalance to the excessive concentration on minutia

Dr Rundles raised the question of the relationship of these myeloproliferative and leukemic states to plasma cell leukemia and multiple myeloma This question of multiple myeloma and from there to the lymphomatous disorders reminds me of a comment of G K Chesterton's who wrote a book in response to a challenge

*Somebody on top of a bus on which he was traveling one day said to him* pointing to a passerby If a man believes in himself he will go far"

Chesterton replied That is just the sort of thing he ought not to believe in

His companion asked Then what should he believe in?

Chesterton remarked I will go home and write a book about it which he did

He says in the preface to the book I wrote this book in response to a challenge and he has only to throw me another challenge and I will write another book

I have connected up in this tentative scheme this morning these myeloproliferative and aplastic states and these tuberculous leukemoid reactions and I am prepared to tie in the lymphomatous group on the other side of the ring but I think we had *better have our lunch instead*

Finally I think Dr Doan mentioned that in his experience some of these onsets in aplasia—the aplastic state onset—have been followed by a monocytic type of response I have seen this in at least two cases but my own experience with the development of these states is not large enough to make any general comment

As far as the French literature is concerned they don't seem to have developed monocytic relations but myeloblastic or lymphoblastic ones almost irrespectively

# 31

# Nucleic Acids and Related Derivatives as Targets for Chemotherapy

GEORGE BOSWORTH BROWN and M EARL BALIS
Sloan Kettering Division Cornell University Medical College
New York New York

The concept that nucleic acids occupy a key position in the economy of the cell has led to numerous attempts to control growth and multiplication by interference with their synthesis or metabolism The theorizing" that this approach could be useful has received support from the fact that empirical testing has succeeded in showing that certain related compounds can indeed lead to inhibitions of growth

In view of the apparent importance of nucleic acids to cellular processes it is perhaps not surprising that organisms can biosynthesize their nucleic acids from simple and generally available metabolites In the course of their synthesis *de novo* the purines are assembled from small units such as glycine CO the one carbon unit "formate" and "$NH_3$" the final closure of the purine ring is completed several steps after the attachment of ribose and phosphate (26) In other words nucleic acid synthesis is not dependent on any external supply of the large units which make up the polynucleotides Despite the fact that they need not be supplied it has been found that a number of purines pyrimidines and/or their ribose derivatives can be utilized when exogenous supplies are available (12) Our present conception of pathways leading to purines of nucleic acids is outlined by the very simplified flow sheet shown in Fig 1 (12) The first purine derivative that appears the "common intermediate" in Fig 1 is a nucleotide of a purine

The available evidence suggests that it is nucleotides which are formed in the tissues however despite the fact that free purines are not intermediates on the pathways of biosynthesis of these nucleotides mechanisms exist for the incorporation of exogenously supplied purines The

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be utilized with remarkable efficiency and can therefore greatly spare that synthesis This is particularly well demonstrated in some micro organisms (3 5), and is significant in mammals (17)

*That every tissue studied and presumably each cell, is capable of* synthesizing purines *de novo* has been suggested by the results of the incorporation of small precursors into various tissues in several animals This capability has also been demonstrated *in vitro* with slices of various tissues (27) cell suspensions (2) including ascites cells (23) and leu kocytes (19) and of course with many microorganisms (1 3 5) *In vivo* experiments have demonstrated that neoplastic tissues possess this ability to a marked degree *in fact, even when purines are supplied* exogenously the tumor uses them to a decidedly smaller extent than do other tissues (6) Unfortunately tumors are not so unique in this respect as might be desired since other rapidly growing tissues such as in testines regenerating liver and bone marrow have a similar tendency With an emphasis on synthesis *de novo* in tumors it is logical that inter ference with the biosynthetic mechanisms leading to the formation of purine derivatives can lead to a preferential toxicity to these tissues This has indeed been found to be true and Drs Goldthwait (16) and Buchanan (13) will be discussing those processes wherein certain agents with demonstrated efficacy the antifolic acids and azaserine are operat ing

Variations of the incorporations of exogenously supplied purines from tissue to tissue are also to be considered Although Fig 1 shows only adenine and guanine there are a number of other purines which can similarly be incorporated The utilizations of four purines (including hypoxanthine and 2 6 diaminopurine) and of glycine for instance into various organs of the hamster show significant differences in their rela tive incorporations (6) Such differences are not yet interpretable but do point to some biochemical distinctions between tissues

Some very impressive documentations (12) of great diversity in pat terns of purine utilization is found in the differences in the direction and facility for interconversions of adenine and guanine derivatives in several species (Fig 2) At one extreme (the rat *Torula utilis*) adenine is readily transformed into guanine derivatives but the converse does not occur At the other extreme (*Lactobacillus leichmannii Tetrahymena pyri formis*) the converse predominates that is guanine is readily trans formed into adenine derivatives but adenine is not transformed into guanine derivatives At the middle of the species "spectrum" are ex amples of continuous interconversion of these two purines (*Lactobacillus casei*) There are also more conservative organisms (*Escherichia coli*)

"active adenine and "active guanine" in this scheme represent nucleotide derivatives which can be further anabolized into the polynucleotides Thus not only does a nucleotide arise through the common pathway of synthesis but the free purines are initially converted into nucleotides

This flow sheet also accounts for the transformations of either exogenously supplied adenine or guanine into polynucleotide derivatives of the other There is evidence which suggests that cells can interchange the substituent groups but that the purine ring is maintained essentially intact throughout the conversions (3 24)

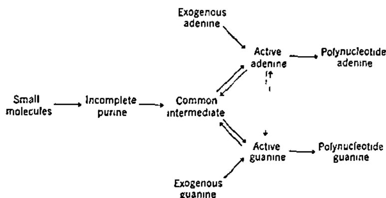



*FIG 1*

An interconversion of the purines should be possible through simple reversal of the steps leading from the common intermediate but this is not necessarily so or at least there is an alternate pathway Recently an *Aerobacter aerogenes* mutant obtained by Drs Brooke and Magasanik (10) offered an opportunity for a rather crucial test of this point (1) This mutant requires guanine for growth and when so grown excretes a large amount of xanthosine into the medium By growth and tracer studies it was possible to demonstrate that this mutant was blocked in the synthesis of guanine from the common intermediate and that the xanthosine shunted into the medium was arising by synthesis *de novo* presumably from an intermediate which was failing to reach the guanine derivative The organism still was able to convert the exogenously supplied guanine into adenine derivatives and the existence of some alternate pathway for the interconversion of adenine and guanine derivatives is demonstrated

It is obvious from this scheme that exogenously supplied purines can compete with those arising biosynthetically In some instances they can

guanine which has been strongly emphasized by Bennett and Skipper who tested a large series of tumors An appreciable conversion of 2 6 diaminopurine into tumor nucleic acid guanine is to be particularly noted in view of the metabolic relationships of diaminopurine and guanine There is also a small utilization of hypoxanthine in a number of tumors

From the point of view of chemotherapy it is to be expected that the more complex the substrate antagonized the fewer will be the processes affected If processes particularly important to a given cell can be attacked a greater selectivity may be possible With the recognition that there are pronounced quantitative differences between the purine metabolisms of various normal tissues and of tumors it can be hoped that preferential interferences with specific metabolic steps of purine metabolism can be a fruitful approach As knowledge about metabolic pathways and the function of known antimetabolites accumulates it may become possible to take intentional advantage of that knowledge in developing antimetabolites At present it is possible only to suggest general types of compounds From scores or hundreds of examples meticulous testing must find those which may prove to be effective

There is no *a priori* reason for a purine analog to be an antagonist Since free purines have not been allotted any place in the metabolic schemes there should be nothing with which such an antagonist can compete There is a fortunate lack of specificity of the enzymes which are involved in the incorporation of purine bases into the more complex molecules however and there is evidence that several inhibitors exert their effects only after they have been metabolized to more complex derivatives

There are several unnatural compounds which have apparently been anabolized into large molecules With one of these notably 8 aza guanine there has been a clear demonstration that it can be incorporated into nucleic acids in lieu of guanine Its ribose phosphate derivatives can be isolated from TMV synthesized in its presence (25) two aza g[illegible] s presumably the 2 and 3 isomers have been de[illegible] [illegible]r a similar incorporation of the pyrimidine 2 [illegible] for the incorporation of 5 bromouracil into [illegible]ucleic acids (28) In such cases the poly[illegible] base could fail to perform its proper [illegible]abolite"

[illegible] found in nature is converted to [illegible]tes for several enzymes It is not [illegible] a nucleotide has been dem[illegible]

which have the ability to interconvert in either direction but which favor the direct utilization of the exogenous purine when it is in limited supply From the limited data available for man on the incorporation of purines into leukemic white cells both *in vivo* and *in vitro* (19) it appears that man s position is intermediate in this species "spectrum"

2 6 Diaminopurine is another purine which is readily utilized by all species tested It is always transformed into the usual nucleic acid purines predominantly into polynucleotide guanine sometimes even

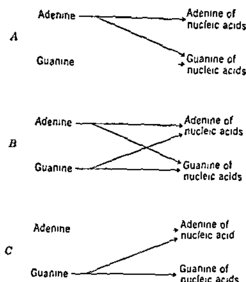



FIG 2

more readily than is exogenous guanine It is transformed into adenine derivatives to a degree similar to that observed for exogenous guanine in the particular species In connection with this biochemical similarity of diaminopurine and guanine it should be pointed out that each carries functional groups in the 2 and 6 positions

When the limited data now available on the incorporation of exogenous purines into various tumors (6 8) is considered one can already recognize some characteristics in addition to the generally limited incorporations of preformed purines There are numerous small differences in the relative incorporations of the several purines tested but like the differences between organs of the host these are at the stage where they can be tabulated but not discussed Certain characteristics sufficiently consistent to be noteworthy include a low incorporation of

*guanine which has been strongly emphasized by* Bennett and Skipper who tested a large series of tumors An appreciable conversion of 2,6-diaminopurine into tumor nucleic acid guanine is to be particularly noted in view of the metabolic relationships of diaminopurine and guanine There is also a small utilization of hypoxanthine in a number of tumors

From the point of view of chemotherapy it is to be expected that the more complex the substrate antagonized the fewer will be the processes affected. If processes particularly important to a given cell can be attacked a greater selectivity may be possible With the recognition that there are pronounced quantitative differences between the purine metabolisms of various normal tissues and of tumors it can be hoped that preferential interferences with specific metabolic steps of purine metabolism can be a fruitful approach As knowledge about metabolic pathways and the function of known antimetabolites accumulates it may become possible to take intentional advantage of that knowledge in developing antimetabolites At present it is possible only to suggest general types of compounds From scores or hundreds of examples meticulous testing must find those which may prove to be effective

There is no *a priori* reason for a purine analog to be an antagonist Since free purines have not been allotted any place in the metabolic schemes there should be nothing with which such an antagonist can compete There is a fortunate lack of specificity of the enzymes which are involved in the incorporation of purine bases into the more complex molecules however and there is evidence that several inhibitors exert their effects only after they have been metabolized to more complex derivatives

There are several unnatural compounds which have apparently been anabolized into large molecules With one of these notably 8-aza guanine there has been a clear demonstration that it can be incorporated into nucleic acids in lieu of guanine Its ribose phosphate derivatives can be isolated from TMV synthesized in its presence (25) two aza guanine nucleotides presumably the 2- and 3-isomers have been de tected There is evidence for a similar incorporation of the pyrimidine 2 thiouracil into TMV (21) and for the incorporation of 5-bromouracil into certain bacterial deoxyribonucleic acids (28) In such cases the poly nucleotide containing the abnormal base could fail to perform its proper function and be the true "antimetabolite"

Diaminopurine which has not been found in nature is converted to derivatives which can serve as substrates for several enzymes It is not only incorporated into a nucleotide—such a *nucleotide has been dem*

which have the ability to interconvert in either direction but which favor the direct utilization of the exogenous purine when it is in limited supply From the limited data available for man on the incorporation of purines into leukemic white cells both *in vivo* and *in vitro* (19) it appears that man s position is intermediate in this species "spectrum"

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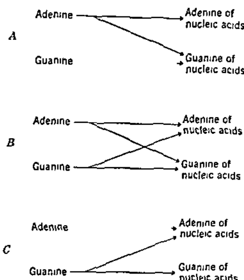



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more properly be considered as a competitor of the transformation of a hypoxanthine derivative into either guanine or adenine derivatives If the "common intermediate is formulated as a hypoxanthine derivative and if 6-mercaptopurine is incorporated into an analogous derivative by the same enzyme which incorporates hypoxanthine the competition between these two derivatives could lead to the observed results

The toxicity of diaminopurine can be blocked by 6-mercaptopurine and vice versa and these facts can be correlated with the above postulations *The 6-mercapto compound would be an analog of both the adenine* derivative which is converted to a coenzyme and of the diaminopurine intermediate which leads to the "2 amino coenzyme" The 6-mercapto derivative could completely inhibit the synthesis of the adenine metabolite from adenine or the toxic 2 amino derivative from diaminopurine and the organism would make the coenzyme by an alternate route similar to that used by the diaminopurine resistant mutant The reversal of the mercaptopurine toxicity by diaminopurine could be due to *diaminopurines serving as the source of an additional supply of the* natural purines

One of the most toxic purine derivatives studied is the riboside of purine itself Studies of its metabolism have shown that it can be converted to the adenine and guanine of polynucleotides but that this compound is not incorporated unchanged (18) The position of entry of purine riboside into the metabolic scheme is uncertain but it involves the replacement of a hydrogen in the 6-position by a functional group *possibly in analogy to the similar replacement of a hydrogen in the 2* position which occurs in the conversion of adenine into guanine In addition three nucleotides containing unsubstituted purine appear in the soluble nucleotides of the liver and it is now thought that these are the 5 mono di and triphosphates Here again it is not the formation of abnormal nucleic acids but it may well be the formation of analogs of normal nucleoside phosphates or of coenzymes which is responsible for the toxicity The observation by Biesele (9) that the *toxicity of purine riboside (to tissues in culture) is most effectively* blocked by 5 phosphate derivatives of adenosine can be construed a support of this hypothesis

There has as yet been no demonstration that a purine antimetabolite acts by incorporation into a DNA though this possibility still must be considered The incorporation of at least one purine analog into an RNA has been observed presumably with the production of an abnormal nonfunctioning RNA If RNA is a cytoplasmic factor responsible for cellular heredity and is the metabolically active "working" nucleic

onstrated in the acid soluble fraction from rat liver (31)—but it is further transformed into a guanine derivative which in turn reaches the poly nucleotide. Transformation into adenine derivatives which also occurs may be via the guanine derivative which must be formed but the possibilities for a more direct pathway are not excluded Just prior to the original observation that it is a metabolite (7) diaminopurine was found (14) to be a potent antimetabolite There is a score of systems in which it leads to marked inhibitory effects and in most instances the inhibitory action has been shown to be blocked by adenine (11) In a cooperative study with Hitching and Elion an extensive series of experiments was carried out on *L casei* and a diaminopurine resistant mutant of it [1] There was no obvious influence of diaminopurine on poly nucleotide synthesis under appropriate conditions it could even supply all the purine requirement for guanine and adenine of the polynucleotides

The general tendency of organisms is to convert diaminopurine into guanine at the same time diaminopurine exerts an inhibitory action by competition with adenine These facts are rationalized through the observation that the same enzyme which incorporates adenine into a nucleotide is sufficiently nonspecific to carry out the initial incorporation of diaminopurine into an analogous derivative On one hand the purine moiety of this diaminopurine derivative is transformed into guanine On the other hand the diaminopurinenucleotide moiety is built by another nonspecific enzyme into something which is effectively a 2 amino analog of an adenine derivative with which it is then in competition It is postulated that this product is some essential adenine containing nucleo tide coenzyme This concept receives additional support from the find ing that the adenosinekinase which will phosphorylate adenosine in the 5 position will similarly phosphorylate diaminopurine riboside In fact in the proper enzyme system a triphosphate derivative a 2 amino ATP can be formed (22)

Another antimetabolite which can apparently be metabolized to a nucleotide is 6 mercaptopurine Despite its similarity in structure to adenine it does not seem to compete directly with adenine derivatives xanthine is a more effective reversing agent (20) In the diaminopurine resistant mutant of *L casei* which is characterized by extensive loss of the ability to incorporate adenine or diaminopurine 6 mercaptopurine is still able to exert its inhibitory activity The results of Elion *et al* (15) and subsequent tracer studies (4) suggest that 6 mercaptopurine can

1 The results of this work have been summarized (11)

24 Marrian, D H Spicer V L Balis M E and Brown G B *J Biol Chem* **189** 533 (1951)
25 Matthews R E F *J Gen Microbiol* **10** 521 (1954)
26 Reichard P in "The Nucleic Acids (E Chargaff and J N Davidson eds.) Vol. 2, p 277 Academic Press New York 1955
27 Reichard, P and Bergstrom S *Acta Chem Scand* **5** 190 (1951)
28 Wacker A Trebst A Jacherts D and Weygand F *Z Naturforsch* **9b** 616 (1954)
29 Warburg, O *Science* **123** 309 (1956)
30 Weinhouse S *Advances in Cancer Research* **3** 269 (1955)
31 Wheeler G P and Skipper H E *J Biol Chem* **205** 749 (1953)

acid it should be a favorable target There are the several lines of evidence which suggest that coenzymes are susceptible to agents affecting purine metabolism Since an imbalance of aerobic and anaerobic glycolysis has been implicated in the neoplastic process (29 30) it is not unreasonable to expect that the balance of coenzymes regulating electron and proton transfer may be more uniquely sensitive in such tissues and the often ignored possibilities of antimetabolite interference with the purine moieties of nucleotide coenzymes deserve more emphasis

The patterns of incorporation of purines into RNA seem to mirror qualitatively their incorporation into DNA and the purines of the coenzymes must arise from the same purine supplies Thus the anabolic patterns of RNA which are the most accessible for study may serve as a guide to chemotherapeutic interferences with any of the three general purine containing targets

## REFERENCES


1 Balis M E Brooke M S Brown G B and Magasanik B *J Biol Chem* **219** 917 (1956)
2 Balis M E and Dancis J *Cancer Research* **15** 603 (1955)
3 Balis M E Levin D H Brown G B Elion G B VanderWerff H and Hitchings G H *J Biol Chem* **196** 729 (1952)
4 Balis M E Levin D H Brown G B Elion G B Nathan H and Hitchings G H *J Biol Chem* submitted for publication
5 Balis M E Levin D H and Luzzati D *J Biol Chem* **216** 9 (1955)
6 Balis M E Van Praag D and Brown G B *Cancer Research* **15** 673 (1955)
7 Bendich A Furst S S and Brown G B *J Biol Chem* **185** 423 (1950)
8 Bennett L L Jr Skipper H E Stock C C and Rhoads C P *Cancer Research* **15** 485 (1955)
9 Biesele J J Slautterback M C and Margolis M *Cancer* **8** 87 (1955)
10 Brooke M S and Magasanik B *J Bacteriol* **68** 727 (1954)
11 Brown G B in "Antimetabolites and Cancer (C P Rhoads ed ) p 285 American Association for the Advancement of Sciences 1955
12 Brown G B and Roll P M in "The Nucleic Acids" (E Chargaff and J N Davidson eds ) Vol 2 p 341 Academic Press New York 1955
13 Buchanan J M this volume p 523
14 Elion C B and Hitchings G H *J Biol Chem* **187** 511 (1950)
15 Elion G B Singer S and Hitchings G H *J Biol Chem* **204** 35 (1953)
16 Goldthwait D A this volume p 555
17 Goldthwait D A and Bendich A *J Biol Chem* **196** 841 (1952)
18 Gordon M P and Brown G B *J Biol Chem* **220** 927 (1956)
19 Hamilton L D *Nature* **172** 457 (1953)
20 Hutchison D J *Ann N Y Acad Sci* **60** 212 (1954)
21 Jeener R and Rosseels J *Biochim et Biophys Acta* **11** 438 (1953)
22 Kornberg A and Pricer W F Jr *J Biol Chem* **193** 481 (1951)
23 LePage G A *Cancer Research* **13** 178 (1953)

32

# Uracil Antagonists As Inhibitors of Transplantable Tumors

GEORGE H HITCHINGS
Chemotherapy Division The Wellcome Research Laboratories
Tuckahoe New York

The search for exploitable biochemical disparities between tumor tissue on the one hand and normal tissue on the other so far has not revealed any major biochemical disunities such diversities as have been found have generally been small quantitative differences In the field of nucleic acid derivatives some differences between the majority of tumors and normal tissues in the utilization of guanine have been reported by Bennett *et al* (1) and it is possible that some quantitative differences in the utilization of hypoxanthine may account for the antineoplastic activity of 6 mercaptopurine (5) Recently Rutman *et al* (13) have called attention to striking disparities in the incorporation of uracil, between liver and hepatoma Leibman and Heidelberger (11) have pursued this observation and studied the incorporation of labeled uracil into the Flexner Jobling carcinoma and normal tissues in the rat These workers found that contrary to the results of earlier investigations (12, 13) uracil was incorporated in some normal tissues as well as in the carcinoma The incorporation appeared to be related to the rate of growth and was most prominent in intestinal epithelium

In any case the possible existence of an important quantitative difference in uracil incorporation between tumor and most normal tissues suggested a reinvestigation of the problem of the effects of uracil antagonists on transplantable neoplasms

Our own investigation of uracil antagonists began over a decade ago with a study of the effects of alterations of the functional groups in the uracil structure (9) The replacement of one or both of the hydroxyl groups of uracil by mercapto groups produces antagonists The competitive relationship between thiouracil and uracil was studied by Strandskov and Wyss (16) In addition to alterations in functional

will be observed that in both the folic acid containing medium (PFA) and in the thymine containing medium (PT) isobarbituric acid is competitive with uracil

Studies of this kind led to the selection of a number of more or less potent uracil antagonists It was of interest to study these for their possible inhibitory effects on transplantable rodent tumors Table I shows the results of some of these studies It will be seen that barbituric acid thiouracil the hydrazinopyrimidine and orotic acid are compounds

TABLE I
EFFECTS OF URACIL ANALOGS ON SARCOMA 180

| Compound | Dose mg/kg | Effect | |
|---|---|---|---|
| Barbituric acid | 600 | — | |
| 2 6-Dithiouracil | 250 | — | |
| 2-Hydrazino-4 hydroxy pyrimidine | 500 | — | |
| 2 Thioorotic acid | 500 | — | |
| Isobarbituric acid | 500 | — | † |
| 2-Thiouracil | 500 | ± — | † |
| 2 Thiocytosine | 125 | — | † |
| | 250 | — | † |
| 2 6-Dichloropyrimidine | 125 | — | † |
| | 250 | — | † |
| 6 Azauracil | 125 | — | † |
| | 500 | — | † |
| 6-Azacytosine | 125 | ± — | † |

Negative data (see references 14 and 15)
† Unpublished experiments with D A Clarke and C C Stock

which gave negative results (14 15) on sarcoma 180 The results of hitherto unpublished studies with other uracil antagonists on the same tumor are also given Isobarbituric acid at the highest level tested (100 mg/kg) had no inhibitory effect Of the various uracil antagonists tested only thiouracil in one instance and 6 azacytosine in one instance gave as much as a plus-minus inhibition It appeared unlikely that uracil antagonism would provide a means for a broad attack on transplantable tumors Nevertheless further investigations of uracil antagonism were undertaken

Early work had dealt chiefly with efforts to prepare antagonists by modifications of the functional groups of uracil itself keeping the pyri

groups per se the possibility of producing uracil antagonists by the ad dition of further functional substituents in the 5 and 6 positions of the pyrimidine moiety was investigated For the most part the introduc tion of additional substituents in the 6 position such as hydroxyl methyl and amino had the effect of diminishing the activity of uracil of uracil like compounds and of uracil antagonists The introduction of additional functional groups in the 5 position however produced compounds with very striking effects (7 9) Thus 5 bromouracil was found to be an an tagonist of thymine under some conditions but to simulate thymine

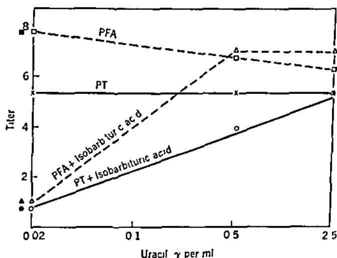



Fig 1 Isobarbituric acid as an antagonist of uracil in the growth of *Lactobacillus casei* Growth is represented by the titer (ordinate) The media are those used previously (8)

under special conditions A very large literature has grown up in recent years around the incorporation of bromouracil moieties in place of thy mine in the deoxyribonucleic acids of various species (3 17 18) The introduction of a nitro group produced a folic acid antagonist 5 Amino uracil was a sort of hybrid folic acid and thymine antagonist (8)—at least it failed to give clear cut competitive results with either of the nutrilites 5 Hydroxyuracil (isobarbituric acid) was found to be a rather strong inhibitor the action of which at first was somewhat mystifying since its inhibition was not reversed by either thymine or folic acid But on investigation it was found to be a competitive antagonist of uracil (7) This is shown in Fig 1 which depicts the growth of *Lactobacillus casei* in the presence and absence of isobarbituric acid and uracil It

cytosine in *L casei* Approximately 5 γ of azauracil or 250 γ of aza cytosine produces nearly complete inhibition of this organism in a medi um in which uracil is absent The addition of uracil restores growth in a competitive fashion (Fig 4) The results with sarcoma 180 previously referred to showed no very pronounced activity with either one of these compounds although there was a suggestion of activity with azacytosine When the same compounds were tested against adenocarcinoma 755 however quite different results were obtained This perhaps is shown

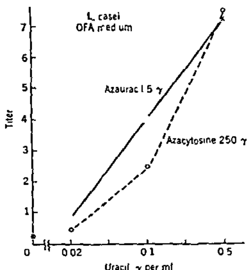



FIG 4 6-Azapyrimidines as antagonists of uracil in the growth of *Lactobacillus casei* Growth is represented by the titer (ordinate) The media are those used previously (8)

most clearly by reference to Table II which gives the results of treat ment of tumor bearing animals with various levels of 6-azauracil It will be observed that 125 mg/kg showed an apreciable inhibition of tumor growth which was increased at a dosage of 250 mg/kg At 500 mg/kg however the tumor growth was completely suppressed and all the ani mals recovered from the tumor This is quite a striking result, since in our experience only one or two other compounds for example 6 mer captopurine produce effects of a similar magnitude

Thus although uracil antagonism must be regarded as a means of tumor inhibition which is not general it is capable of producing interest ing results in at least one specific transplantable tumor It will be of

midine ring intact A new group of uracil antagonists of some interest has now been prepared by alteration of the ring system itself Of particular interest are the 1 2 4 triazines (6 azapyrimidines) (4) Interest in these was aroused by the finding that the asymmetrical triazine analog (10) of the pyrimidine antimalarial *pyrimethamine* (6) *as well as the*

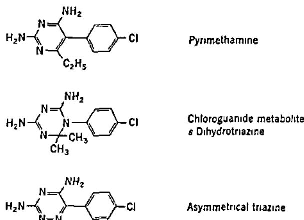



Fig 2 Compounds with antimalarial activity

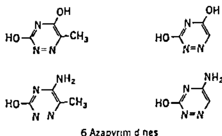



Fig 3 Asymmetrical triazines analogous to natural pyrimidines

symmetrical dihydrotriazine (2) is rather active as an antimalarial Thus the relationship suggested by the rather obvious structural analogy (Fig 2) finds expression in the biological effects also

For this reason the syntheses of pyrimidine analogs in both the asymmetrical and symmetrical triazine series have been undertaken The pertinent 6 azapyrimidines (*as* triazines) are shown in Fig 3 These and a number of interesting intermediates are being studied currently 6 Azauracil has been found to be a competitive antagonist of uracil Strangely enough azacytosine appears to be an antagonist of uracil rather than of

# 33

# The Specific Action of Azaserine on Enzymes Concerned with Purine Biosynthesis[1]

JOHN M BUCHANAN BRUCE LEVENBERG IRVING MELNICK and STANDISH C HARTMAN

Division of Biochemistry Department of Biology
Massachusetts Institute of Technology Cambridge Massachusetts

There have been many instances in previous investigations where the use of metabolic inhibitors has been of great value in clarifying complex physiological responses or a sequence of biochemical reactions In a general way these compounds have been classified into two main categories competitive and noncompetitive inhibitors Iodoacetic acid and fluoride are two outstanding examples of the latter type Iodoacetic acid serves as an alkylating agent of sulfhydryl groups on enzymes i e triose phosphate dehydrogenase (21) Inhibition by iodoacetate permitted physiologists to demonstrate that muscular contraction depended on the breakdown of phosphate containing compounds (phosphocreatine and ATP) and that the formation of lactic acid was associated only indirectly with contraction (28) Fluoride is thought to inhibit enzymes by virtue of its ability to act as a complexing agent of the divalent cations Ca and Mg which are essential activators of certain enzymes as enolase and many phosphatases (29) Noncompetitive inhibition is therefore characterized by an interaction of inhibitor with some component of the enzyme system and not involving the substrate

A third metabolic inhibitor of importance malonic acid is the classical example of an inhibitor of the competitive type It was shown over twenty years ago by Quastel and Wooldridge (30) that malonic acid

[1] This work has been supported by grants-in aid from the National Cancer Institute National Institutes of Health United States Public Health Service the National Science Foundation and the Damon Runyon Memorial Fund for Cancer Research

interest to test other uracil antagonists and further members of the tri azine series and to explore their effects on a spectrum of transplantable tumors

TABLE II

EFFECTS OF 6-AZAURACIL ON ADENOCARCINOMA 755

(In each experiment 6 tumor bearing animals were treated with the dose indicated 6 times in 10 days starting on the eighth day after implantation The tumors were dissected and weighed on the twenty first day The number index represents the number of tumors found at the end of the experiment the size index is the ratio of weights of experimental to control tumors )

| Dose mg /kg | Tumor index | | Toxic deaths |
|---|---|---|---|
| | Number | Size | |
| 125 | 6/6 | 0 75 | 0 |
| 250 | 5/6 | 0 40 | 0 |
| 500 | 0/6 | 0 0 | 1/6 |

3 5-Dihydroxy 1,2 4 triazine

REFERENCES

1 Bennett L L Jr Skipper H E Stock C C and Rhoads C P *Cancer Research* 15 485 (1955)
2 Carrington H C Crowther A F and Stacey G J *J Chem Soc* 1017 (1954)
3 Dunn D B and Smith J D *Nature* 174 305 (1954)
4 Falco E A Pappas E and Hitchings G H *J Am Chem Soc* 78 1938 (1956)
5 Hitchings G H *Am J Clin Nutrition* 3 321 (1955)
6 Hitchings G H *Trans Roy Soc Trop Med Hyg* 46 467 (1952)
7 Hitchings G H Elion G B and Falco E A *J Biol Chem* 185 643 (1950)
8 Hitchings G H Elion G B Falco E A Russell P B and VanderWerff H *Ann NY Acad Sci* 52 1318 (1950)
9 Hitchings G H Falco E A and Sherwood M B *Science* 102 251 (1945)
10 Hitchings G H Maggiolo A Russell P B VanderWerff H and Rollo I M *J Am Chem Soc* 74 3200 (1952)
11 Leibman K C and Heidelberger C *Federation Proc* 14 243 (1955)
12 Plentl, A and Schoenheimer R *J Biol Chem* 153 203 (1944)
13 Rutman R. J Cantarow A and Paschkis K E *Cancer Research* 14 119 (1954)
14 Stock C C *Cancer Research* Suppl 1 (1953)
15 Stock C C Barclay R K Furst A Gellhorn A Lester J and Shimkin M B *Cancer Research* Suppl 2 (1955)
16 Strandskov F B and Wyss O *J Bacteriol* 50 237 (1945)
17 Weygand F Wacker A and Dellwag, H *Z Naturforsch* 7b 19 (1952)
18 Zamenhof S and Griboff G *Nature* 174 307 (1954)

cause the regression of the Crocker sarcoma 180 in mice (39) and extend the life span of mice suffering from certain types of leukemia (9). A point of additional interest is that there is a potentiation of effect in some cases where azaserine and 6 mercaptopurine are administered together (9).

During the last few years we have carried out a program in this laboratory concerned with testing the effects of antimetabolites on an enzymatic system which synthesizes inosinic acid from its elementary precursors—glycine, formate, bicarbonate, glutamine, aspartic acid, ribose 5 phosphate and ATP. Our own interest in azaserine was stimulated by the observation of Skipper and his colleagues (35) that administration of azaserine to mice reduced the incorporation of radioactive glycine and formate into tissue nucleic acids. At the suggestion of Dr Alexander Moore of the Mellon Institute we carried out preliminary trials with this compound with the view in mind that it might interfere with enzymatic reactions concerned with the incorporation into inosinic acid of formyl groups which are metabolically derived from serine. Such, however, was not found to be the case and further attempts to relate azaserine to competitive inhibition of serine metabolism were discouraged.

On testing azaserine for its effect on inosinic acid synthesis with extracts of pigeon liver it was found that inosinic acid formation was completely inhibited at relatively low concentrations of the antibiotic. Certain intermediates of purine biosynthesis accumulated in considerably larger amounts than in the absence of azaserine. These compounds were isolated and identified as glycinamide ribotide (GAR) and α (N formyl) glycinamide ribotide (FGAR) (20). When the latter compound was incubated with the accessory substrates—aspartic acid, bicarbonate, glutamine and formate and pigeon liver enzymes—inosinic acid was readily formed. In the presence of low concentrations of azaserine synthesis was drastically reduced. When the concentration of aspartic acid, bicarbonate and glutamine was increased one at a time it was found that the synthesis of inosinic acid from formylglycinamide ribotide could be partially restored by the addition of glutamine at higher concentrations. At this higher concentration of glutamine synthesis could again be reduced by increasing the azaserine concentration (Fig 1). These experiments were the first indication that azaserine was behaving as an inhibitor of inosinic acid biosynthesis by virtue of its action as an analog of glutamine. The structural relationship of glutamine and azaserine is shown in Fig 2.

inhibits the conversion of succinic to fumaric acid by action on the enzyme system succinic dehydrogenase Moreover the degree of inhibition is not related to the concentration of malonic acid alone but to the ratio of the concentration of succinic and malonic acids Malonic acid is sufficiently similar to succinic acid in structure to combine reversibly with the active site on the enzyme This inhibitor was invaluable in studying the Krebs tricarboxylic acid cycle

The principle of competitive inhibition has been an important concept in the development of the field of intermediary metabolism An interesting early observation was that of Woods (43 44) who showed that the bacteriostatic effect of sulfanilamide is competitively reversed by *p* aminobenzoic acid a compound not previously known to possess any biochemical function It was suggested that the sulfonamide a structural analog of *p* aminobenzoic acid served as a competitive inhibitor of a bacterial enzyme system for which *p* aminobenzoic acid was an essential cofactor This search for structural analogs of metabolites has been extended with considerable success by Shive and his co workers (42) Using structural analogs they have been able to relate essential cofactors to specific functions in microbial metabolism

Analog metabolite inhibition has had widespread application in the development of cancer chemotherapy Special emphasis has been placed on the synthesis of analogs of cofactors purines pyrimidines or amino acids in the belief that the formation and development of new cells whether cancerous or normal are primarily dependent on the synthesis of nucleic acids and proteins and on the cofactors involved in these processes The development of cancer research along these lines received its impetus from the investigations of Farber *et al* (10) who demonstrated that aminopterin was an antagonist of folic acid and as such increased the life span of children suffering from acute leukemia Since this report folic acid has been recognized as a cofactor specifically involved in both amino acid metabolism and nucleic acid synthesis

A whole host of antimetabolites have been synthesized and tested for their chemotherapeutic effects in both tumors and leukemia Among the most important of these may be listed 2 6 diaminopurine (6) 8 azaguanine (2) and 6-mercaptopurine (7 36) In some cases compounds isolated from natural sources have had beneficial effects as antimetabolites Azaserine (O diazoacetyl L serine) is an outstanding example of a compound isolated as an antibiotic and later found to be effective as an antimetabolite in tumor development and in leukemia Although the results of the effects of this compound have not been communicated in full preliminary reports indicate that administration of azaserine will

cause the regression of the Crocker sarcoma 180 in mice (39) and extend the life span of mice suffering from certain types of leukemia (9). A point of additional interest is that there is a potentiation of effect in some cases where azaserine and 6 mercaptopurine are administered together (9).

During the last few years we have carried out a program in this laboratory concerned with testing the effects of antimetabolites on an enzymatic system which synthesizes inosinic acid from its elementary precursor—glycine, formate, bicarbonate, glutamine, aspartic acid, ribose-5 phosphate, and ATP. Our own interest in azaserine was stimulated by the observation of Skipper and his colleagues (35) that administration of azaserine to mice reduced the incorporation of radioactive glycine and formate into tissue nucleic acids. At the suggestion of Dr Alexander Moore of the Mellon Institute we carried out preliminary trials with this compound with the view in mind that it might interfere with enzymatic reactions concerned with the incorporation into inosinic acid of formyl groups which are metabolically derived from serine. Such, however, was not found to be the case, and further attempts to relate azaserine to competitive inhibition of serine metabolism were discouraged.

On testing azaserine for its effect on inosinic acid synthesis with extracts of pigeon liver, *it was found that inosinic acid formation was completely inhibited* at relatively low concentrations of the antibiotic. Certain intermediates of purine biosynthesis accumulated in considerably larger amounts than in the absence of azaserine. These compounds were isolated and identified as glycinamide ribotide (GAR) and α (N formyl) glycinamide ribotide (FGAR) (20). When the latter compound was incubated with the accessory substrates—aspartic acid, bicarbonate, glutamine and formate and pigeon liver enzymes—inosinic acid was readily formed. In the presence of low concentrations of azaserine synthesis was drastically reduced. When the concentration of aspartic acid, bicarbonate and glutamine was increased one at a time it was found that the synthesis of inosinic acid from formylglycinamide ribotide could be partially restored by the addition of glutamine at higher concentrations. At this higher concentration of glutamine synthesis could again be reduced by increasing the azaserine concentration (Fig 1). These experiments were the first indication that azaserine was behaving as an inhibitor of inosinic acid biosynthesis by virtue of its action as an analog of glutamine. The structural relationship of glutamine and azaserine is shown in Fig 2.

## Enzymatic Steps Concerned with the Biosynthesis of Inosinic Acid

In order to discuss further experiments on azaserine to be presented in a later section a brief summary is made here of the work which has been carried out in connection with the enzymatic biosynthesis of inosinic acid An understanding of the metabolic precursors of inosinic

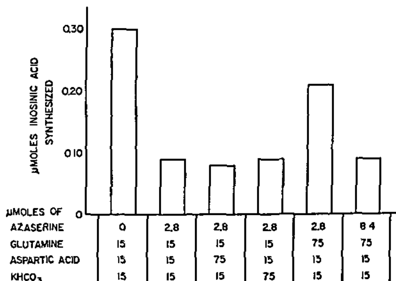



Fig 1 Ability of L glutamine to overcome the inhibitory action of L azaserine in the synthesis of IMP from FGAR

acid is a prerequisite for enzymatic studies Through a series of studies beginning in 1946 (5 25 33 37 38) it was shown (Fig 3) that glycine is the precursor of carbon atoms 4 and 5 and nitrogen atom 7 of the purine ring CO is the precursor of carbon atom 6 formate of carbon atoms 2 and 8 glutamine (amide N) of nitrogen atoms 3 and 9 and aspartic acid of nitrogen atom 1 Ribose 5 phosphate should also be

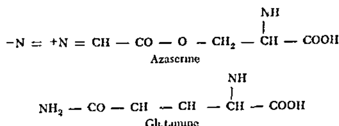



Fig 2 Structural similarities between azaserine and glutamine

added to this list of precursors since inosinic acid is the first complete purine structure to be formed *de novo* (16) and this requires that some metabolic precursors should be ribotide compounds

By isolation of individual enzymatic components from avian liver it has been possible to link these precursors together through a series of reactions and intermediates which account for the step by step formation of inosinic acid (Fig 4) References have been made at the individual steps of the synthesis to the investigators who have been concerned with their description Certain features of the scheme still need further clarification Although synthetic 5 phosphoribosylamine is required for synthesis of glycinamide ribotide from glycine and ATP (14)

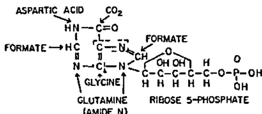



Fig 3 Precursors of inosinic acid

at the time of this writing it has not been possible to isolate this compound from the reaction of 5 phosphoribosylpyrophosphate and glutamine These latter two compounds react with each other yielding glutamic acid and pyrophosphate (14)

GAR may be formylated to FGAR by several reactions Formate the $\beta$ carbon of serine and carbon atom 2 of inosinic acid are all precursors of the "active formyl compounds which directly donates the one carbon unit

The structure of 5 amino 4 imidazole (N succinylocarboxamide) ribotide (SAICAR) is at present tentative There are certain features of its chemical properties which must be resolved before this structure can be taken as final Until the enzyme which splits this new intermediate (SAICAR) into its products is freed of the enzyme fumarase it will not be possible to determine whether fumurate or malate is the actual compound formed

5 Formamido 4 imidazolecarboxamide has been formed chemically from 5 amino 4 imidazolecarboxamide ribotide and formic acid and may be converted into inosinic acid by action of the newly discovered enzyme inosinicase To date however it has not been possible to demonstrate

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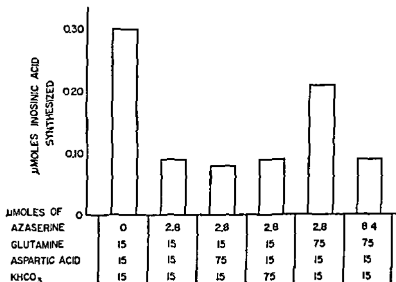



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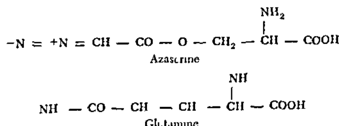



FIG 2 Structural similarities between azaserine and glutamine

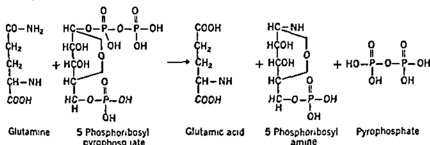
the accumulation of this compound when 5 amino-4 imidazolecarbox amide is reacted with serine and TPN and a compound of the folic acid series This latter reaction is a complicated process involving at least three individual enzymes These enzymes have not been freed sufficiently of inosinicase which is active in low concentrations and which converts the 5 formamido-4 imidazolecarboxamide ribotide almost quantitatively into inosinic acid

### Location of the Site of Azaserine Inhibition

*With the individual steps of purine biosynthesis de novo thus de*scribed it has been possible to determine the step at which azaserine exerts its affect as an inhibitor

TABLE I

Reaction




| Vessel | Azaserine µM | Glutamine µM | Glutamic acid formed mµM |
|---|---|---|---|
| 1 | 0 | 1 | 124 |
| 2 | 1 | 1 | 103 |
| 3 | 10 | 1 | 71 |

In Tables I to V is shown the effect of azaserine on five of the most representative steps of this biosynthetic process As would be anticipated from the earlier experiments azaserine inhibited the enzymatic reaction concerned *with the formation of FGAM from FGAR glutamine and* ATP This enzymatic step was inhibited at relatively low concentrations of the antibiotic Other reactions were inhibited to a limited extent but only at much higher concentrations of azaserine It is noteworthy that the other step at which glutamine is involved (PRPP + glutamine → glutamic acid + inorganic pyrophosphate + PRA) is not seriously affected by azaserine It is concluded therefore that the effect of aza

FIG 4 Enzymatic reactions in the synthesis of inosinic acid

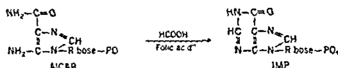
serine on the *de novo* synthesis of purines is entirely due to its inhibition of the conversion of FGAR to FGAM

### Properties of the Enzyme

The enzyme responsible for the conversion of FGAR to FGAM has been purified from chicken and from pigeon liver approximately fifty fold The reaction requires glutamine Mg and K ions and ATP (Table

TABLE V

Reaction




| Vessel | Azaserine µM | IMP formed mµM |
|---|---|---|
| 1 | 0 | 63 |
| 2 | 1 | 65 |
| 3 | 25 | 69 |

TABLE VI

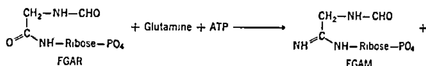
| System | FGAM mµM |
|---|---|
| Complete | 49 |
| —ATP | 1 |
| —K | 24 |
| —Mg | 1 |
| —Glutamine | 4 |
| —FGAR | 1 |

VI) Glutamic acid ADP and inorganic phosphate are products of the reaction

The assay for the above enzyme is a relatively simple procedure The enzyme system is incubated for a short time (5 to 10 minutes) with the appropriate substrates and the reaction is stopped by heating the vessel in a boiling water bath for 1 minute The contents of the flask are then reacted with a second enzyme which converts FGAM to 5-aminoimidazole ribotide (AIR) This latter compound is an arylamine which may be determined by a colorimetric procedure according to the method of Bratton and Marshall (3)

## TABLE II

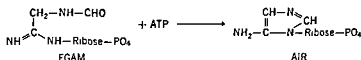
Reaction




| Vessel | Azaserine μM | Glutamine μM | FGAM synthesized from FGAR mμM |
|---|---|---|---|
| 1 | 0 | 2 | 17.2 |
| 2 | 0.2 | 2 | 6.7 |
| 3 | 2.0 | 2 | 0.9 |

## TABLE III

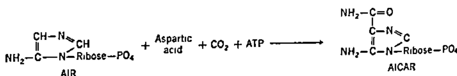
Reaction




| Vessel | Azaserine μM | Glutamine μM | AIR synthesized from FGAM mμM |
|---|---|---|---|
| 4 | 0 | 0 | 24.8 |
| 5 | 0.2 | 0 | 23.8 |
| 6 | 2.0 | 0 | 15.0 |
| 7 | 2.0 | 3 | 16.8 |

## TABLE IV

Reaction




| Vessel | Azaserine μM | AICAR formed mμM |
|---|---|---|
| 1 | 0 | 56 |
| 2 | 2 | 60 |

and involved factors other than a simple competitive relationship between the inhibitor and glutamine

In Fig 5 experiments are reported in which the concentrations of glutamine and azaserine are varied over a hundredfold range but the ratio of their concentrations is kept constant at a value of 10 At the lowest range of metabolite and inhibitor concentrations the reaction was inhibited 50% At the higher concentrations the inhibition of reaction was 69% of the uninhibited controls This relative constancy of inhibition over such a broad range of concentration of substances is further evidence of the competitive relationship of these two compounds

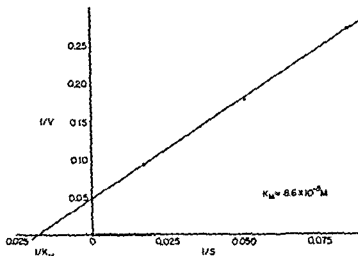



FIG 6 Determination of the $K_M$ value of FGAR in the reaction FGAR → FGAM

In a third series of experiments analysis of the kinetics of the reaction have been made according to the classical procedures of Michaelis and Menton and Lineweaver and Burk From the data shown in Fig 6 calculation has been of the $K_M$ value of FGAR This value is approximately $1 \times 10^{-4}$ M In order to determine the $K_M$ value of glutamine and the $K_I$ value of azaserine experiments were carried out in which the enzyme was incubated with excess FGAR and varying amounts of glutamine In accordance with the method of Lineweaver and Burk 1/V was plotted against 1/S at three different levels of azaserine concentration (Fig 7) The intercepts of the resulting curve with the abscissa gives the value of the apparent $K_M$ ($K_P$) at the different levels of inhibitor

## Mechanism of Azaserine Inhibition of the Enzyme FGAR → FGAM

The competition between azaserine and glutamine for the enzyme FGAR → FGAM is well demonstrated in Table VII and Fig 5 As shown by the data obtained in these experiments the degree of inhibition of the enzyme reaction is a function of the ratio of the concentration of

TABLE VII

| Azaserine μM | Glutamine μM | FGAM mμM |
|---|---|---|
| — | 2 | 56 5 |
| 0 15 | 2 | 26 0 |
| 0 15 | 4 | 38 0 |
| 0 15 | 6 | 44 0 |
| 0 15 | 8 | 46 5 |
| 0 15 | 10 | 46 5 |

glutamine to azaserine At low ratios inhibition of reaction is virtually complete As the ratio of glutamine to azaserine increases the extent of inhibition decreases (Table VI) At a ratio of substrate to inhibitor of approximately 18 the degree of inhibition is 50% In experiments so far concluded it has not been possible as yet to restore completely the *full* enzymatic activity of the system by increasing the glutamine concentration These preliminary results indicated that possibly the inhibition of the reaction by azaserine was more complicated than initially supposed

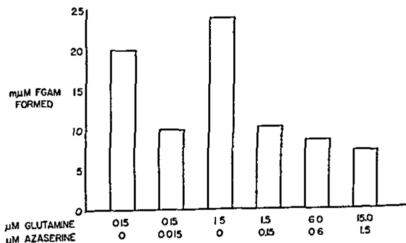



Fig 5 The inhibition of reaction as a function of the ratio of glutamine to azaserine over a large range of concentrations of materials

serine inhibition are still obscure and that the significance of the values will have to undergo re-examination. Since these constants are some measure of the relative potency of azaserine as an inhibitor of this reaction they may have some practical value.

The characteristics of the data shown in Fig 7 also confirm previous ideas concerning the nature of the inhibition of azaserine. According to the comments of Friedenwald and Maengwyn-Davies (13) these data indicate that the elements of both competitive and noncompetitive inhibition are involved in the action of azaserine on the enzyme. The non-

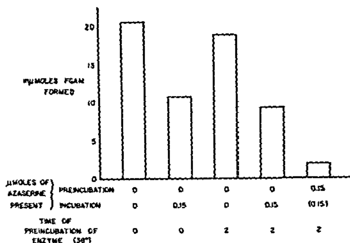



FIG 9 Effect of preincubation with azaserine on the activity of the enzyme system responsible for the conversion of FGAR to FGAM. All vessels were incubated for 5 minutes at 38° with the following components of the basic system: 0.10 μM FGAR, 2 μM glutamine, 2 μM ATP.

competitive aspects of azaserine inhibition can be demonstrated by experiments in which the enzyme is preincubated with azaserine (with and without glutamine) prior to addition of the other substrates of the main incubation system, i.e. FGAR and ATP. As shown in Fig 9 preincubation of the enzyme for so short a time as 2 minutes with a low amount of azaserine results in such damage to the enzyme that little or no reaction takes place on addition of the other substrates (including glutamine) and the main incubation carried out for 5 minutes. Similar preincubation of the enzyme in the absence of substrates or inhibitor did not significantly affect its activity or the effect of azaserine on this activity during the main incubation period.

When these values for $K_P$ in turn were plotted against inhibitor concentration a $K_M$ value of $6.15 \times 10^{-4}$ M for glutamine and a $K_I$ value of $3.4 \times 10^{-5}$ M for azaserine were obtained (Fig 8) The ratio of $K_M$ to $K_I$ is 18 a value which indicates that azaserine is a fairly potent inhibitor of the reaction These kinetic data are presented with the realization that the theoretical interpretations behind the mechanism of aza

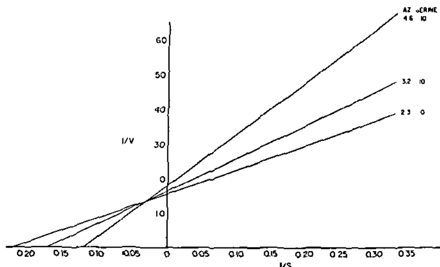



FIG 7 Lineweaver and Burk plot showing type of inhibition

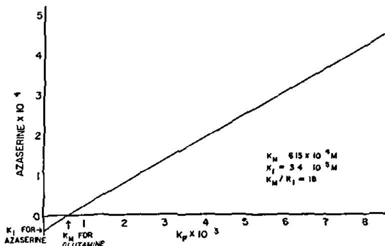



FIG 8 Determination of $K_I$ and $K_M/K_I$ for azaserine

that extracts of *E coli* which had assimilated formate $C^{14}$ in short term experiments in the presence of azaserine accumulated significant amounts of $C^{14}$ labeled formylglycinamide riboside and the ribotide Only at much higher concentrations of azaserine was there an interference of amino acid metabolism Their opinion is the same as ours—that the primary site of azaserine inhibition *in vivo* is interference of purine synthesis *de novo*

The understanding of the mechanism of azaserine inhibition of this system could have some practical implications in the method of administering this compound clinically Possibly both the therapeutic and toxic effects of azaserine may be due to its irreversible and pronounced effect on the enzyme FGAR → FGAM In view however of the role of glutamine in preventing inhibition of azaserine it might be of interest to determine whether both the beneficial and detrimental effects of azaserine could be controlled by administering it with varying amounts of glutamine so that both compounds glutamine and azaserine reach the intracellular enzyme sites at the same time Glutamine administered after azaserine would probably play little role since glutamine behave as a protective agent and cannot reverse the detrimental effects of the inhibitor once they have been allowed to take place

The enzymatic approach to the study of the mechanism of action of drugs and antimetabolites could assume increasing importance now that many of the fundamental reactions of intermediary metabolism have been studied and elucidated The report by Feigelson *et al* (11) that azaguanine inhibits adenosine metabolism by combining irreversibly with the enzyme adenosine deaminase is another example of the use which can be made of enzymatic systems in the understanding of the action of chemotherapeutic agents

The studies reported herein were primarily responsible for directing attention to the fact that azaserine is a competitive inhibitor of glutamine Since that time other analogs of glutamine have been synthesized which are much more potent inhibitors of both physiological systems and the enzymatic reaction described above The correspondence of physiological and enzymatic response is striking and offers further evidence for the usefulness of the enzymatic method as a means of screening rapidly a large series of compounds whose relationship as an antimetabolite to a given metabolite might be suspected

## REFERENCES

1 Abrams R and Bentley M *J Am Chem Soc* 77 4179 (1955)
2 Armistead C C Jr Burchenal J H Karnofsky D A and Southam C M *Cancer* 2 1087 (1949)

In a last series of experiments shown in Fig 10 a study was made on the effect of glutamine in protecting the enzyme against the damage produced by azaserine during this preincubation The enzyme in all vessels of this experiment was preincubated for 2 minutes in the presence of varying combinations of inhibitor and glutamine In the control vessel to which no inhibitor was added either during the preincubation or incubation period 39 mμM of FGAM were found If azaserine was included only in the incubation period the synthesis of FGAM was reduced to 23 5 mμM If on the other hand as shown in column 3 the aza

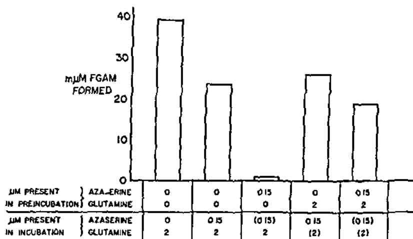



Fig 10 Protection by glutamine of inhibition caused by azaserine Basic system added for incubation 0 1 μM FGAR 2 μM ATP

serine was added during the preincubation period but the glutamine not included until later the synthesis dropped to 1 mμM When as shown in the last column glutamine was included in the vessel with azaserine during the preincubation period the amount of FGAM formed was 19 mμM —a value considerably greater than 1 and only slightly less than 23 5

These data demonstrate that glutamine acts essentially by competing with azaserine for an enzyme site and thus prevents the enzyme from reacting in (what now seems) an irreversible manner with the inhibitor In the absence of glutamine azaserine apparently reacts rapidly with the enzyme causing its inactivation

These findings could possibly have some importance in the understanding of how azaserine behaves toward the intact physiological system The interesting observation has been made by Tomisek *et al* (40)

35 Skipper H E Bennett L L Jr and Schabel, F M Jr *Federation Proc* 13 298 (1954)
36 Skipper H E Thomson J R Elion G B and Hitchings G H *Cancer Research* 14 294 (1954)
37 Sonne J C Buchanan J M and Delluva A M *J Biol Chem* 166 395 (1946)
38 Sonne J C Lin I and Buchanan J M *J Am Chem Soc* 75 1516 (1953)
39 Stock, C C Reilly H C Buckley S M Clarke D A and Rhoads C P *Nature* 173 71 (1954)
40 Tomisek A J Kelly H J and Skipper H E *Abstr 128th Meeting Am Chem Soc* (Minneapolis) p 5C (September 1955)
41 Warren L, and Flaks J G *Federation Proc* 15 379 (1956)
42 Williams R J Eakin R E Beerstecher E Jr and Shive W "The Biochemistry of B Vitamins Reinhold Publishing Corp New York 1950
43 Woods D D *Brit J Exptl Pathol* 21 74 (1940)
44 Woods D D and Fildes P *Chemistry & Industry* 59 133 (1940)

3 Bratton A C and Marshall E K Jr *J Biol Chem* **128** 537 (1939)
4 Buchanan J M and Schulman M P *J Biol Chem* **202** 241 (1953)
5 Buchanan J M and Sonne J C *J Biol Chem* **166** 781 (1946)
6 Burchenal J H Karnofsky D A Kingsley Pillers E M Southam C M Myers W P L Escher G C Craver L F Dargeon H W and Rhoads C P *Cancer* **4** 549 (1951)
7 Burchenal J H Murphy M L Ellison R R Sykes M P Tan T C Leone L A Karnofsky D A Craver L F Dargeon H W and Rhoads C P *Blood* **8** 965 (1953)
8 Carter C E and Cohen L H *J Am Chem Soc* **77** 499 (1955)
9 Clarke D A Philips F S Stock C C Elion G B and Hitchings G H *Abstr 125th Meeting Am Chem Soc* (Kansas City) 12M (March 1954)
10 Farber S Diamond L K Mercer R D Sylvester R F Jr and Wolf J A *New Engl J Med* **238** 787 (1948)
11 Feigelson P Wu M S L and Davidson J D *Abstr 128th Meeting Am Chem Soc* (Minneapolis) p 5C (September 1955)
12 Flaks J G and Buchanan J M *J Am Chem Soc* **76** 2275 (1954)
13 Friedenwald J S and Maengwyn Davies G D *in* 'The Mechanism of Enzyme Action (W D McElroy and B Glass eds ) p 154 The Johns Hopkins Press Baltimore 1954
14 Goldthwait D A Greenberg G R and Peabody R A *Biochim et Biophys Acta* **18** 148 (1955)
15 Goldthwait D A Peabody R A and Greenberg G R *J Am Chem Soc* **76** 5258 (1954)
16 Greenberg G R *J Biol Chem* **190** 611 (1951)
17 Greenberg G R *Federation Proc* **12** 211 (1953)
18 Greenberg G R *J Am Chem Soc* **76** 1458 (1954)
19 Greenberg G R Jaenicke L and Silverman M *Biochim et Biophys Acta* **17** 589 (1955)
20 Hartman S C Levenberg B and Buchanan J M *J Am Chem Soc* **77** 501 (1955)
21 Hellerman L *Physiol Revs* **17** 454 (1937)
22 Kornberg A Lieberman I and Simms E S *J Am Chem Soc* **76** 2027 (1954)
23 Lagerqvist U *Acta Chem Scand* **9** 1028 (1955)
24 Levenberg B and Buchanan J M *J Am Chem Soc* **78** 504 (1956)
25 Levenberg B Hartman S C and Buchanan J M *Federation Proc* **14** 975 (1955)
26 Lieberman I *J Am Chem Soc* **78** 251 (1956)
27 Lukens L N and Buchanan J M *Federation Proc* **15** 305 (1956)
28 Lundsgaard E *Biochem Z* **217** 162 (1930)
29 Meyerhof O Lohmann K and Schuster P *Biochem Z* **286** 301 (1936)
30 Quastel J H and Wooldridge W R *Biochem J* **22** 689 (1928)
31 Remy C N Remy W T and Buchanan J M *J Biol Chem* **217** 885 (1955)
32 Schulman M P and Buchanan J M *J Biol Chem* **196** 513 (1952)
33 Shemin D and Rittenberg D *J Biol Chem* **167** 875 (1947)
34 Shive W Ackermann W W Gordon M Getzendaner M E and Eakin R E *J Am Chem Soc* **69** 725 (1947)

# 34

# Nucleotide Metabolism and Cancer Chemotherapy

HOWARD E. SKIPPER
Kettering Meyer Laboratory (Affiliated with Sloan Kettering Institute)
Southern Research Institute Birmingham Alabama

There appear to be two theoretical approaches to the elimination of the leukemias as medical problems (1) prevention of the "mutagenic" events which result in leukemic cells and (2) development of means for long term repression of leukemic cell division or the outright destruction of these neoplastic cells in their numerous sites of sequestration without excessive damage to vital normal cells It is this latter approach which will be discussed

Currently there are two major research approaches to the problem of attempting to find agents which might be of value in control of cancer (1) empirical screening of large numbers of materials against various animal and human neoplasms (2) searches for exploitable biochemical differences between normal and neoplastic cells with the ultimate objective of designing and synthesizing metabolite antagonists with greater selective toxicity to cancer cells There is much emotional, shortsighted and unproductive debate on the relative merits of these two approaches *Both efforts are obviously complementary* I am tempted to take this dogmatic statement as my text for this discussion

You have already heard Dr Buchanan ably discuss his group's fundamental work with regard to the effect of azaserine on *de novo* purine synthesis It is of interest to note that this inhibitor which has been a useful tool in adding to our knowledge of the chemical events leading to purine nucleotides (4) was picked out of a crude beer and synthesized because of its inhibitory action toward sarcoma 180 (9) We postulated that azaserine might be an inhibitor of *de novo* purine biosynthesis because of its potentiation of the antitumor activity of 6-mercaptopurine (3) and the potentiating activity of this combination against L 1210 leukemia (Table I)

inhibition of *E coli* Wheeler and Brockman (unpublished data) have observed that pyridoxal phosphate will prevent azaserine inhibition of *E coli* There is growing evidence that the $B_6$ coenzyme is involved in the conversion of formylglycinamide ribotide to formylglycinamidine ribotide perhaps by activation of one of the reactants leading to the latter compound

Now let me quickly review the evidence for the site of blockade of azaserine within the chemical events leading to purine nucleotides by means of a metabolic map (Fig 1) Much of the fundamental work indicated on this chart came from the results of Buchanan and his associates This map includes data obtained in a pigeon liver enzyme system in *E coli* and in the intact animal

We have observed that 6-mercaptopurine inhibits labeled formate glycine and hypoxanthine but not adenine incorporation into the polynucleotide purines of the intact animal (6) Thus it now seems reasonable to assume that the potentiated anticancer activity observed in animals resulting from therapy with azaserine plus 6-mercaptopurine or amethopterin plus 6-mercaptopurine may be the result of a sequential blockade Such a hypothesis is of conceivable value in the search for effective combination chemotherapy Certainly the fundamental information on the intermediates en route to nucleotide synthesis and knowledge of the nitrogen donors provides new targets for potential antimetabolite synthesis Thus fundamental biochemistry has helped to provide some possibly useful information for the chemotherapist and I believe all would agree that the work of Stock *et al* (9) leading to the discovery of azaserine has materially aided fundamental biochemistry

Having tried to make this point I should like to spend a few minutes talking about some preliminary studies which we are carrying out in collaboration with Dr C P Rhoads and his associates in an attempt to gain information on exploitable biochemical differences between normal and neoplastic tissues Such knowledge is badly needed in any rational effort to synthesize more effective anticancer agents

These studies entail the injection of selected labeled compounds into animals bearing either animal or human tumors and comparing their relative incorporation into the DNA or RNA of the tumors versus various host normal tissues I do not have the time or the inclination to review all the results obtained to date (1 2) but I should like to refer to one rather profound difference which we have observed and some studies which suggest a possible explanation for this apparent difference between cancer and normal tissues In some twenty different types of animal tumors and two different human tumors studied to date it has been

Just previous to the observation of the azaserine 6-MP potentiation we had observed the potentiating antileukemic activity of amethopterin (an inhibitor of *de novo* purine synthesis) and 6-MP (8). Tracer experiments in the intact animal quickly showed that azaserine treatment inhibited incorporation of formate and glycine into nucleic acid purines but failed to inhibit adenine incorporation (7). Hartman *et al.* (4) then found the exact site of azaserine blockade in their pigeon liver enzyme system capable of *de novo* synthesis of inosinic acid. Tomisek, Kelly

TABLE I
EFFECTS OF AZASERINE PLUS 6-MERCAPTOPURINE ON LIFE SPAN OF MICE WITH L-1210 LEUKEMIA

| Treatment | Average life span | |
|---|---|---|
| | Days | Per cent above control |
| None | 6.6 | |
| X† | 10.5 | 59 |
| Y‡ | 10.6 | 61 |
| X + Y | 20.4 | 209 |
| X/2 + Y/2 | 21.4 | 224 |
| X/4 + Y/4 | 15.1 | 128 |

* This combination was first shown to be potentiating by Clarke *et al.* (3).
† X = azaserine 8.0 mg./kg. × 10.
‡ Y = 6-mercaptopurine 40 mg./kg. × 10.

and Skipper (unpublished data) have confirmed this site of blockade in isolated intact cells (*E. coli*) capable of *de novo* synthesis of purine nucleotides and purine-containing coenzymes. Bennett, Schabel, and Skipper (unpublished data) have shown that azaserine inhibits *de novo* purine synthesis *in vivo* without affecting nucleic acid thymine synthesis and by use of labeled precursors which enter the pathway to purine nucleotides at different sites have delimited the site of blockade to the area between the points of entry of glycine and 5-amino-4-imidazole-carboxamide. This observation is, of course, in agreement with the results obtained with isolated systems (4; Tomisek *et al.*, unpublished data). We have also observed that azaserine inhibits *de novo* nucleic acid purine synthesis *in human tumors growing in cortisonized hamsters* (Bennett *et al.*, unpublished data). Then, if more confirmation is needed regarding the site of purine blockade by azaserine, Schabel has noted that glutamic acid, glutamine, 5-amino-4-imidazolecarboxamide, hypoxanthine, adenine, guanine, or xanthine will partially prevent azaserine

periments) (2) a deficiency within the tumor cells of catalysts responsible for conversion of guanine to guanosine or guanylic acid (this appears unlikely in view of the finding that sarcoma 180 incorporates guanylic acid 8-$C^{14}$ poorly when compared to normal mouse tissues) and (3) the possibility that tumors contain relatively high levels of guanase and therefore rapidly degrade guanine before it can be incorporated into tumor nucleic acids It is data on this last possibility that I should like to present at this time

It has been shown that AIC inhibits deamination of 8-azaguanine *in vitro* (5) and a possibly similar enzyme is inhibited by AIC *in vivo* (5) Therefore it seemed of importance to determine the effects of AIC on guanine incorporation into tumor polynucleotides under the conditions of previous experiments The results of experiments designed to shed light on the effects of AIC (guanase inhibition?) have been carried out by Bennett and associates (unpublished data) The results of these efforts are summarized in Table II

These results seem quite suggestive that sarcoma 180 (and by inference other neoplastic cells) is indeed high in guanine deaminase activity We should like to know whether this possible high guanase level is associated with uncontrolled growth in neoplasia and even more we should like to know how this difference might be exploited to the disadvantage of disseminated cancer cells Perhaps one might find an innocuous purine derivative which guanase would convert to a cytotoxic agent and thus exploit the possibly higher guanase activity of tumors *in vivo* Or it might be possible to block guanine synthesis in both neoplastic and normal cells and reverse this block preferentially in normal tissues with guanine or guanylic acid Obviously the kind of biochemical differences we have seen to date are not what we are searching for Theoretically for a rational approach to the selective poisoning of disseminated neoplastic cells one needs knowledge of an obligate biochemical pathway for neoplastic cells which is unimportant to normal mammalian cells such as is apparently the case with pathogenic bacteria which are selectively poisoned *in vivo* by sulfonamides

I should now like to talk briefly about screening results recently obtained on a new class of purine analogs the pyrazolo(3 4 *d*)pyrimidines Working with Dr Roland Robins we have now screened some sixty pyrazolo(3 4 *d*)pyrimidines and I believe have obtained some interesting structure activity relationships It is of no little comfort to us that the same compounds out of this rather large series have "fallen out" in two different experimental screening procedures (*1*) inhibition of growth of adenocarcinoma 755 in C57 black mice and (*2*) subcutaneous

consistently observed that tumors fail to incorporate guanine into their polynucleotides as well as do normal tissues This difference is no small one but is of the order of five to one hundred fold

When biochemical situations which might account for this difference were considered the following seemed plausible (1) differential cell permeability (this now appears unlikely as the result of preliminary ex

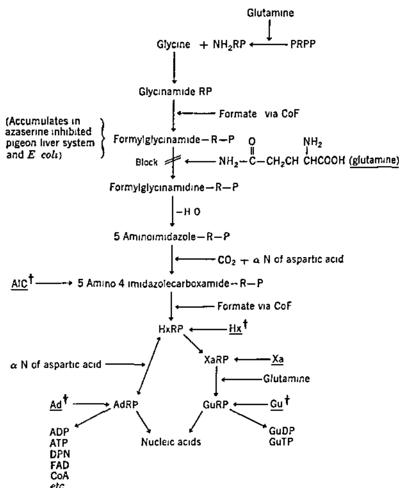


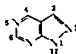

FIG 1 Underlined compounds have been shown to prevent the inhibition of *E coli* by azaserine An asterisk indicates labeled compounds whose incorporation into nucleotide and polynucleotide purines is inhibited by azaserine A dagger indicates labeled compounds whose incorporation into nucleotide and polynucleotide purines is not inhibited by azaserine Abbreviations P phosphate R ribose AIC 5 amino-4 imidazolcarboxamide Hx hypoxanthine Ad adenine Xa xanthine Gu guanine

growth or life span experiments with leukemia 5178 in DBA mice. The results obtained to date which indicate a relationship between structure and anticancer activity are summarized in Table III.

From the theoretical standpoint it is interesting to note that two active 4-aminopyrazolo(3,4-*d*)pyrimidines are blocked in the 1 position with a methyl group and therefore have no obvious point for attachment of ribose phosphate *in vivo*.

TABLE III

PYRAZOLO(3,4-*d*)PYRIMIDINES WHICH HAVE SHOWN ANTICANCER ACTIVITY TO DATE




| Substituent on pyrazolo-(3,4-*d*)pyrimidine | Activity against Adenocarcinoma 755 | Activity against Leukemia 5178 |
|---|---|---|
| 4-Amino | + | + |
| 4-Methylamino | + | + |
| 4-Dimethylamino | + | + |
| 4-Ethylamino | | ? |
| 4-Isopropylamino | + | + |
| 4-n-Butylamino | ? | ? |
| 4-Furfurylamino | + | |
| 1-Methyl-4-amino | + | + |
| 1-Methyl-4-methylamino | + | + |

Note: + = confirmed statistically significant activity; ? = suggestive activity as yet unconfirmed; blank = not yet run.

It will be of some interest to learn more about the mechanisms of action of these compounds which contain a ring structure isomeric with the purine ring, and to determine if they possess antileukemic activity in man.

One more subject. It has long been of concern to all of us just what the stumbling blocks to chemotherapeutic "cure" of leukemia are. It is generally suspected that the existence of drug resistant mutants in untreated populations of leukemic cells which eventually overgrow in spite of therapy is one factor, and, of course, the failure yet to uncover an agent or a combination of agents with sufficient selective toxicity to neoplastic cells must be another.

TABLE II

EFFECTS OF AIC ON UTILIZATION OF GUANINE BY SARCOMA 180 AND NORMAL TISSUES OF THE MOUSE

(Groups of 10 animals were employed and tissues were pooled for polynucleotide purine isolation. AIC • HCl was administered at 200 mg/kg 1 hour before and again immediately before injection of labeled guanine. All animals were sacrificed 6 hours after injection of labeled guanine.)

| | Specific activities (counts/sec γ purine) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sarcoma 180 | | | | Intestine | | | | Liver | | | |
| | DNA | | RNA | | DNA | | RNA | | DNA | | RNA | |
| | Ad | Gu | Ad | Gu | Ad | Gu | Ad | Gu | Ad | Gu | Ad | Gu |
| Control | <0.02 | <0.03 | <0.02 | <0.02 | <0.02 | 0.16 | <0.05 | 0.36 | <0.05 | 0.21 | <0.04 | 0.42 |
| Treated | <0.03 | 0.17 | <0.03 | 0.27 | <0.02 | 0.45 | <0.06 | 0.64 | <0.06 | 0.39 | <0.05 | 0.57 |
| Control | <0.04 | <0.03 | <0.01 | <0.04 | <0.02 | 0.18 | <0.04 | 0.37 | <0.05 | <0.1 | <0.1 | 0.20 |
| Treated | <0.04 | 0.33 | <0.02 | 0.45 | <0.02 | 0.45 | <0.05 | 0.76 | <0.05 | 0.24 | <0.05 | 0.46 |

TABLE IV (*Continued*)

RELATIONSHIP BETWEEN NUMBERS OF LEUKEMIC CELLS (L-4946) INOCULATED AND "CURABILITY" WITH AMETHOPTERIN

| Time of initiation of therapy relative to leukemic inoculation (indicated as 0) hours | Average life span days | Range of life span, days | Number of leukemic deaths | Per cent 68-day cures* |
|---|---|---|---|---|
| | Number of leukemic cells inoculated 1,[illegible]00 | | | |
| Controls | [illegible]3.1 | 19–31 | 10/10 | |
| 0–3 | | | 1/10 | 90 |
| 0 | | | 0/10 | 100 |
| 3 | | | 0/10 | 100 |
| 6 | | | 1/10 | 90 |
| [illegible]4 | | | 0/10 | 100 |
| | | Treated totals | 2/50 | 96 |
| | Number of leukemic cells inoculated 1[illegible]0 | | | |
| Controls | 31.3 | 21–37 | 10/10 | |
| 0–3 | | | 0/10 | 100 |
| 0 | | | 0/10 | 100 |
| 3 | | | 0/9 | 100 |
| 6 | | | 0/10 | 100 |
| [illegible]4 | | | 0/9 | 100 |
| | | Treated totals | 0/48 | 100 |
| | Number of leukemic cells inoculated 12 (ca) | | | |
| Controls | | | 7/10 | – |
| 0–3 | | | 0/10 | 100 |
| 0 | | | 0/10 | 100 |
| 3 | | | 0/9 | 100 |
| 6 | | | 0/10 | 100 |
| 24 | | | 0/10 | 100 |
| | | Treated totals | 0/49 | 100 |

jected intraperitoneally and treatment was by the same route but it should be pointed out that this is not an ascitic form of L-4946 and that the evidence available suggests that such leukemic cells when injected intraperitoneally quickly find their way into the blood stream. The data presented in Table IV appear of some interest. Further work is in progress in an attempt to shed light on the meaning of such results.

It might be of interest from the clinical standpoint to attempt therapy with a drug such as 6-MP (to achieve as complete a remission of acute

We have recently completed a study on the "curability of leukemia L 4946 in highly inbred AK mice under conditions most dissimilar from the spontaneous clinical disease but still of interest to us This experiment shows quite clearly the relationship between numbers of leukemic cells inoculated and the curability under the favorable conditions employed The details of this experiment and results obtained are presented in Table IV The leukemic cells employed in this study were in

TABLE IV

RELATIONSHIP BETWEEN NUMBERS OF LEUKEMIC CELLS (L-4946) INOCULATED AND CURABILITY" WITH AMETHOPTERIN

(The entry 0–3 indicates that the first injection of amethopterin preceded leukemic inoculation by 3 hours In all cases therapy was continued (after the initial treatment) every other day for a total of ten doses Amethopterin was administered uniformly at levels of 3 mg kg intraperitoneally )

| Time of initiation of therapy relative to leukemic inoculation (indicated as 0) hours | Average life span days | Range of life span days | Number of leukemic deaths | Per cent 68 day cures |
|---|---|---|---|---|
| | Number of leukemic cells inoculated | | 1 200 000 | |
| Controls | 13 4 | 12–19 | 10/10 | - |
| 0–3 | 35 3 | 30–57 | 10/10 | 0 |
| 0 | 34 0 | 21–43 | 10/10 | 0 |
| 3 | 34 1 | 27–53 | 10/10 | 0 |
| 6 | 30 8 | 27–35 | 10/10 | 0 |
| 24 | 33 2 | 29–42 | 9/9 | 0 |
| | | Treated totals | 49/49 | 0 |
| | Number of leukemic cells inoculated | | 120 000 | |
| Controls | 16 4 | 14–21 | 10/10 | - |
| 0–3 | 39 6 | 31–59 | 10/10 | 0 |
| 0 | | | 8/10 | 20 |
| 3 | | | 9/10 | 10 |
| 6 | | | 9/10 | 10 |
| 24 | | | 9/9 | 0 |
| | | Treated totals | 45/49 | 8 |
| | Number of leukemic cells inoculated | | 12 000 | |
| Controls | 20 3 | 18–29 | 10/10 | - |
| 0–3 | | | 5/10 | 50 |
| 0 | | | 0/10 | 100 |
| 3 | | | 1/10 | 90 |
| 6 | | | 0/10 | 100 |
| 24 | | | 2/10 | 80 |
| | | Treated totals | 8/50 | 84 |

The most potent agents which can revert the inhibition by azaserine are phenylalanine, tryptophan, and tyrosine. This has been demonstrated by Stock and Kaplan from Sloan-Kettering on studies of inhibition of growth. They imposed a hypothesis that azaserine may be involved in the inhibition of the synthesis of these aromatic amino acids. This is not necessarily so because other substances which are relatives of these aromatic substances also can prevent azaserine inhibition. For example, β-2-thienylalanine, which is an analog of phenylalanine, is potent in reversing the inhibition of azaserine with respect to the synthesis of the imidazoles.

We have no adequate explanation for the effects of the aromatic amino acids, but as a working hypothesis it is possible that azaserine itself is not the true inhibitor but must be metabolized to an inhibitor, and these reversing agents may prevent this activation. This may also be true in the case of glutamine.

I emphasize this only to stress that the ability of a substance to reverse an inhibition does not impose a competitive metabolic function.

Furthermore, we feel that azaserine, although it can be shown to inhibit purine synthesis, must have other functions as far as inhibition of growth is concerned, because purines alone cannot reverse inhibition of growth in the organisms that we use. Glutamine cannot reverse inhibition of growth either. The only substances we have found that can do this are the aromatic amino acids phenylalanine, tryptophan, and tyrosine.

DR. ROBERT GUTHRIE (Buffalo, New York): I should like to extend the remarks of the last speaker and comment particularly on the papers by Drs. Brown and Hitchings, to illustrate two main points deserving of emphasis at this symposium.

First, it has been mentioned in a number of papers here that an antimetabolite can act as a metabolite, depending on the particular cell system or depending on the conditions under which it is tested. In reverse, it is possible for a metabolite to act as an antimetabolite. This has been demonstrated a number of times, but it is possible that this particular point has not received the attention it deserves from those interested in chemotherapy. Studies of nutrition of animals as well as of bacteria have revealed cases of amino acid imbalances. *Internal inhibition—the inhibitory effects of accumulated intermediates on other pathways in the same cell—has been described by Dr. B. D. Davis and others. Another example is the case of the sulfonamide-requiring mutant in Neurospora. The sulfonamide requirement apparently is an expression of the fact that this particular mutant is sensitive not to an unphysiological concentration of p-aminobenzoic acid, but to the "normal" concentration of this vitamin that it itself synthesizes.*

This point is best illustrated by toxopyrimidine, listed *under this name in the* Merck Index. The compound is actually a form of the natural thiamine pyrimidine, 2-methyl-4-amino-5-aminomethylpyrimidine. It was found by Makino *et al.* to be extremely toxic to mice and, further, that the toxicity of this natural pyrimidine was prevented by pyridoxine. More recently Harris has found this same antagonism with the roles of each antagonist reversed. Pyridoxine can interfere with the utilization of the thiamine pyrimidine in synthesis of thiamine in *Neurospora*. Here, then, is a striking example of the point I am stressing in that two metabolites can interfere with each other's action, and either one can act as an antimetabolite, depending on the biological system.

These facts indicate that we might be able to exploit a potential biochemical difference between two cell types to obtain selective toxicity by the proper use of the

leukemia as possible and to get the blast count low) and then to follow immediately with a course of treatment with amethopterin

In closing I should like to reiterate that I believe results obtained in the present screening programs and fundamental biochemical programs are complementary I hope the day will come when knowledge of the biochemistry of cancer the mechanisms of action of antimetabolites and other classes of tumor inhibiting agents and the mechanisms of resistance will prove useful in the search for more effective means for controlling leukemias and other types of cancer

REFERENCES

1 Bennett L L Jr Skipper H E Stock C C and Rhoads C P *Cancer Research* 15 485 (1955)
2 Bennett L L Jr Skipper H E Toolan H W and Rhoads C P *Cancer Research* 16 262 (1956)
3 Clarke D A Philips F S Stock C C Elion G B and Hitchings G H *Abstr 125th Meeting Am Chem Soc* (Kansas City) p 12M (1954)
4 Hartman S C Levenberg B and Buchanan J M *J Am Chem Soc* 77 501 (1955)
5 Mandel H G *in* Antimetabolites and Cancer pp 199 American Association for the Advancement of Science Washington D C 1955
6 Skipper H E *Ann N Y Acad Sci* 60 315 (1954)
7 Skipper H E Bennett L L Jr and Schabel F M Jr *Federation Proc* 13 298 (1954)
8 Skipper H E Thomson J R Elion G B and Hitchings G H *Cancer Research* 14 294 (1953)
9 Stock C C Reilly H C Buckley S M Clarke D A and Rhoads C P *Nature* 173 71 (1954)

## General Discussion

DR JOSEPH S GOTS (Philadelphia Pennsylvania) I should like to recount some of our experiences with azaserindipity "

We are also using microbial systems (*E coli* and *Salmonellae*) We measure the synthesis of the imidazole precursors by using biochemical mutants in systems which do not allow their proliferation We measure the synthetic ability of nonproliferating suspensions of intact cells

Azaserine has a very potent inhibitory action on this synthesis As little as 0 1 γ/ml will cut down the synthesis by 50% and 1 γ/ml will completely inhibit

Glutamine does have some effect in reversing this inhibition by azaserine but it is rather weak and it cannot be divorced from the effect that glutamine itself has in stimulating the synthetic activity in the absence of azaserine No evidence of competition could be demonstrated

hibitory activity than the parent pyrimidine analog and hence azathymine does *not* exert its inhibitory effect by trapping deoxyribose in this manner preventing the biosynthesis of DNA

An interesting relationship was observed between the biological effect of azathymine on the growth of *S faecalis* and its incorporation into the DNA of this microorganism When azathymine-5-$C^{14}$ was added prior to inoculation in a concentration which produced 50% inhibition of growth the incorporation of the radioactive analog into the DNA was of the order of a 15 to 16% replacement of the normal thymine content However when sufficient azathymine-6-$C^{14}$ was added during the logarithmic phase of growth to obtain the same degree of inhibition we found very little incorporation—from zero to less than 1% replacement of thymine Under the conditions where essentially no incorporation of 6-azathymine into the DNA was observed death of all the microorganisms occurred within 48 hours whereas under the conditions of a 16% replacement of DNA thymine no death of the bacterial cells was observed

Hence the mechanism of inhibition of this pyrimidine analog does not appear to be via incorporation into the DNA but more probably by inhibition of the utilization of a required metabolic intermediate or of the function of an essential coenzyme

DR G B BROWN (New York New York) I might comment briefly in regard to what Dr Guthrie was saying

It is well known that there are a number of examples where adenine which is a normal metabolite can be inhibitory at high levels in fact Dr Guthrie had some microorganisms which were inhibited by adenine and in which diaminopurine would reverse the inhibition

I might also add that the purine riboside of which I spoke is a naturally occurring compound It has been isolated so far only from mushrooms but nevertheless we might look for it elsewhere in nature Perhaps its toxicity is an instance of a case of a normal metabolite being present in abnormal concentrations thereby leading to toxicity

I think that normal compounds at abnormal levels is another very very important possibility for interference with biological systems

DR G H HITCHINGS (Tuckahoe New York) I should like to add a few words on the question of unbalanced growth raised by Dr Guthrie and go back to some of the experiments which Dr Gordon reported yesterday You will recall that with one of the pyrimidines of the Daraprim family he got inhibition of bone marrow cellular elements When he tried to protect the bone marrow from the action of this inhibitor by adding a stimulant he got instead increased inhibition

These pyrimidines are primarily inhibitors of cell division which work by preventing the synthesis of the precursors of the nucleic acid necessary for cell division When you stimulate cells by other means what you do essentially is to bring more cells into the state of cell division and so you enhance the activity of the inhibitor

A similar phenomenon has been seen very clearly in the case of Daraprim in malaria where the time of action of the compound has little to do with the method of administration of the compound or the dosage It depends chiefly on the stage in the life cycle of the parasite The rings grow normally in the presence of the compound until it comes time for schizogony and then they disintegrate

natural metabolites themselves This is related to another idea that is currently gaining some attention that is the use of a metabolite and an antimetabolite in combination in which one attempts to increase the selective toxicity between two cell types by protecting one type at the expense of the other I think these ideas may be of particular importance in cancer chemotherapy where the biochemical basis for selective toxicity may be quite subtle and specific

The other point I want to mention is related to Dr Gots comment Although nucleic acid metabolism has received well deserved emphasis as a target of cancer chemotherapy at this conference I am sure many of us would admit the possibility that the anticancer effects of such agents as the antifolics 6 mercaptopurine and azaserine could result from effects on other metabolic pathways involving purines and pyrimidines at catalytic levels far removed from nucleic acid per se

DR A GOLDIN (Bethesda Maryland) I was particularly interested in Dr Skippers last table because we have a similar result with leukemia 1210 in which we have shown a linear relationship between the dose of amethopterin employed and the total number of cells in the leukemic inoculum [*Science* **123** 840 (1956)] I think that is pretty good confirmation of what Dr Skipper showed

I should like to bring out one other point One of the things we have been interested in is the question of protecting against the toxicity of some of these antimetabolites For example we have been able to protect against the toxicity of 6-mercaptopurine with compounds such as adenylic acid guanylic acid and inosinic acid

We put together a combination of sodium formate 4 amino 5 imidazole carboxamide ribose 5 phosphate and citrovorum factor and found that with this we could protect against the toxicity of 6-mercaptopurine We thought this was rather neat especially in view of the work of Buchanan and Greenberg

When we investigated the individual components however we found that although with the same dose for example of ribose 5 phosphate alone we could not get any protection nevertheless if we boosted the dose of ribose 5 phosphate sufficiently without the addition of the other compounds we were able to get protection against 6 mercaptopurine

I was wondering whether this suggests a mobilization of other components in a system *in vivo* in response to introduction of one particular metabolite I wonder whether Dr Skipper or Dr Buchanan might have some comment on that

DR J M BUCHANAN (Cambridge Massachusetts) I dont know whether I exactly understand the question There is one interesting comment that I would make however There is an enzyme that reacts with 6 mercaptopurine and 5 phosphoribosyl pyrophosphate to form the 6 mercaptopurine ribotide This compound was isolated over a year ago in our laboratory

If this is the type of mobilization of which you are speaking then I would agree In other words by introducing the 6-mercaptopurine compound you would get a mobilization of the enzymes which convert it into the ribotide the form in which it is possibly reactive as a chemotherapeutic agent

DR W PRUSOFF (New Haven Connecticut) We have studied the mechanism of action of 6-azathymine the compound discussed by Dr Hitchings and have found that *Streptococcus faecalis* can convert this compound to the corresponding deoxyriboside We find that this nucleoside azathymidine has much more potent in

# 35

## Purine Nucleotide Biosynthesis and Neoplasia[1]

*DAVID A. GOLDTHWAIT*[2]
Western Reserve University, Cleveland, Ohio

The antimetabolites which are most effective in the chemotherapy of leukemia appear to exert their effect on the synthesis of nucleic acids. The most notable of these are 6-mercaptopurine, azaserine, and the folic acid antagonists; other less prominent agents are 2,6-diaminopurine, chloropurine, thioguanine, and azathymine (6). The partial effectiveness of these drugs in the acute leukemia of childhood has made it even more imperative to elucidate the pathways of synthesis of nucleic acids and the factors controlling the rates of the reactions. I wish to discuss some of the earlier steps in the biosynthesis of purine nucleotides on which we have worked, and I shall discuss these with particular reference to the possible control of the rates of these reactions by mechanisms within the normal and neoplastic cell, and also with reference to the action of some antimetabolites.

It was possible to carry out this work for two reasons: first because Dr. Buchanan and his group had defined the ultimate carbon and nitrogen sources of the atoms of the purine ring (4), and second because Dr. Robert Greenberg had developed a soluble enzyme system which would synthesize *de novo* purine nucleotides (13). Much of this work was done in collaboration with Dr. Robert Greenberg and Dr. Richard Peabody, to whom the author is very grateful.

From an acetone powder extract of pigeon liver two aliphatic ribotides were isolated (11, 12) which were precursors of inosinic acid. The structures of these compounds, glycinamide ribotide (GAR) and formyl

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[1] This work was supported by grants from Eli Lilly and Co., the American Heart Association, and the U. S. Public Health Service.

[2] Established Investigator of the American Heart Association.

Dr Robert Guthrie (Buffalo New York) At two different laboratories in two different institutions I have been told that natural purine bases have been found to stimulate the growth of a particular solid tumor (S180) In one case the purine was tested as a control for an experiment in which the purine was combined with amethopterin in the hope of getting a synergistic effect and such a synergistic effect was obtained Amethopterin was more inhibitory to the tumor in combination with the purine These findings would appear to support another basis for combining a metabolite with an antimetabolite If one can make one cell type grow faster than the other and thus push its metabolic machinery faster one can make it more sensitive to the monkey wrench thrown in Further support for this idea could be drawn from consideration of the temperature sensitive mutants of molds and bacteria When the cells are growing faster at higher temperatures a vitamin requirement is exhibited that is not shown at lower temperatures In this case an antimetabolite that interferes with this vitamin might be expected to be more active when growth is faster

# 35

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glycinamide ribotide (FGAR) and their relation to inosinic acid (IMP) are shown in Fig 1[3]

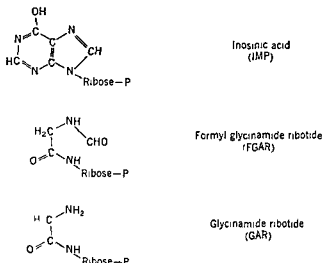



FIG 1

In an analysis of the mechanism of synthesis of glycinamide ribotide the first question which must be raised is What are the substrates required for synthesis? Table I shows that ribose 5 phosphate (R5P) glutamine ATP and glycine react in some manner to form the compound

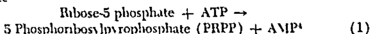
TABLE I
SUBSTRATE REQUIREMENTS FOR GLYCINAMIDE RIBOTIDE SYNTHESIS

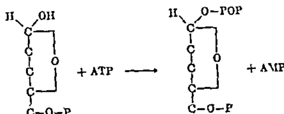
| Substrate | Glycine 1 $C^{14}$ in glycinamide ribotide μM |
|---|---|
| R5P + glutamine + ATP | 0 15 |
| R5P + glutamine | 0 02 |
| R5P + ATP | 0 02 |
| Glutamine + ATP | 0 01 |

The next question is How do these substances react? At the present time we can write three equations and we shall consider these in a step

[3] Abbreviations used in this discussion ATP adenosine triphosphate AMP adenosine monophosphate R5P ribose 5 phosphate PRPP 5 phosphoribosylpyrophosphate PRA 5 phosphoribosylamine $P_i$ inorganic phosphate POI inorganic pyrophosphate GAR glycinamide ribotide FGAR formylglycinamide ribotide THFA tetrahydrofolic acid CF citrovorum factor DPN diphosphopyridine nucleotide

wise fashion The first reaction is the formation of a derivative of ribose 5-phosphate


Ribose-5 phosphate + ATP →
5 Phosphoribosylpyrophosphate (PRPP) + AMP[4] (1)




This reaction is known to be essential because PRPP was shown to substitute for ribose 5 phosphate and an enzyme fraction capable of synthesizing PRPP from ribose 5 phosphate and ATP (10 16) PRPP (19) is a key compound in the synthesis of purine nucleotides It is required in both the *de novo* synthesis and the synthesis of nucleotides from the free bases adenine guanine and hypoxanthine (20) It is reasonable to suspect that an analog of this compound should be able to block purine nucleotide synthesis regardless of the pathway operating within the cell and therefore should accomplish what at present is one of the theoretical aims of combined therapy Practically the design of such an analog may be difficult because of the problem of cellular permeability to the phosphate esters

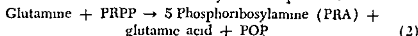
The mechanism of the control of the rate of the reaction by which PRPP is synthesized is obscure Obviously it depends on the amounts of ATP of the enzyme and of ribose 5 phosphate The level of ATP within the cell is important Not only is the total cellular ATP content important but also the location of the ATP in relation to the enzyme involved in PRPP synthesis

Preliminary studies show that at least some of the PRPP synthesizing enzyme resides in the supernatant fraction of a homogenate (78 000 × g for 1 hour) of the pigeon liver It is in this fraction also that the glycolytic enzymes are found which can generate ATP Ribose 5 phosphate may arise by two pathways One is by oxidation of glucose 6 phosphate and 6 phosphogluconic acid to ribose 5 phosphate The alternate pathway is via the transketolase transaldolase series of reactions (17) the enzymes for which are also found in the supernatant fraction Isotopic studies of ribose synthesis in the chick by Bernstein (2) suggest that the latter pathway may be the main mechanism of conversion of glucose to

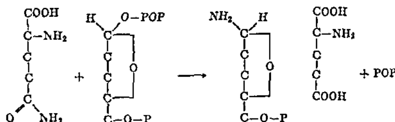
[4] The hydrogens and hydroxyls are omitted for the sake of simplicity

ribose 5 phosphate in the animal It is then clear that the rate of PRPP synthesis depends on the rate of conversion of glucose to ribose 5 phosphate and on the ATP level in the soluble portion of the cytoplasm Is there any difference in this pathway in the normal and in the malignant cell? It is tempting to try to relate this to the observations of Otto Warburg regarding the increased fermentation or glycolytic metabolism and decreased respiratory metabolism in cancer cells as compared to normal cells (29) We may ask Does the increased glycolysis in tumor cells result in an increased conversion of glucose to ribose 5 phosphate which then results in an increase of PRPP synthesis? Is this the primary driving force in nucleotide synthesis? Since nucleotide intermediates do not normally accumulate nor are the levels of purine or pyrimidine nucleotides ever exceptionally high one wonders whether there is some controlling mechanism in the first steps If so does the relationship to glycolysis in some way include a mechanism of control? Needless to say we do not have any chemotherapeutic control of ribose 5 phosphate or PRPP synthesis

The second reaction in the *de novo* synthesis is postulated as follows



Glutamine + PRPP → 5 Phosphoribosylamine (PRA) + glutamic acid + POP (2)




The product of the reaction between PRPP and glutamine is extremely unstable and has not yet been isolated What then is the evidence for 5 phosphoribosylamine (PRA)? Balance studies of the reaction showed that glutamic acid pyrophosphate and ribose 5 phosphate were products It seemed logical that PRA was the initial product which was readily degraded to ribose 5 phosphate PRA was therefore synthesized chemically The material was very unstable at pH 7 0 but would substitute for PRPP and glutamine in the synthesis of glycinamide ribotide (10) (Table II)

Control of the rate of the PRPP glutamine reaction the equilibrium of which lies far toward the product depends on the levels of the substrates PRPP and glutamine Some of the factors involved in PRPP synthesis have been discussed The reactions which maintain the glutamine level

within the cell are multiple and cannot be considered here in detail In mammalian cells a transport mechanism exists for the concentration of glutamine (25) The glutamine levels also depend on the rate of synthesis from glutamic acid ammonia and ATP (24) which is rapid, judging from $N^{15}$ isotopic data (23) One reason for this rapid rate of turn over of $N^{1}$ may be purine nucleotide synthesis for which only the amide nitrogen of glutamine is utilized For example in the PRPP glutamine reaction glutamic acid is regenerated and becomes available again for the synthesis of glutamine Thus any compound which is an antagonist of

TABLE II
SUBSTITUTION OF PRA FOR PRPP AND GLUTAMINE IN GLYCINAMIDE RIBOTIDE SYNTHESIS

| | Glycinamide ribotide formed μM | |
|---|---|---|
| | Experiment 1 | Experiment 2 |
| PRPP + glutamine + ATP | 0 32 | 0 13 |
| PRA + ATP | 1 10 | 0 48 |
| PRA | – | 0 01 |

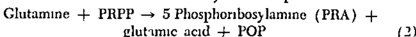
glutamic acid in the synthesis of glutamine such as methionine sulfoxide (28) might be effective in decreasing the intracellular levels of glutamine The effect of such an antagonist would be circumvented by exogenous glutamine however[5] Therefore the effective level of glutamine can be more easily reduced by a glutamine antagonist. Azaserine (26) appears to be such a compound and has been shown by Hartman *et al* to block purine biosynthesis at a step which results in the accumulation of formylglycinamide ribotide (16) In this case azaserine was shown to be a competitive inhibitor of glutamine (5) We have shown that azaserine is also effective as a competitive inhibitor of glutamine in the reaction with PRPP (Table III) The reaction is measured by the liberation of inorganic phosphate from PRPP after incubation with the enzyme glutamine and inorganic pyrophosphatase It is apparent that the inhibition of azaserine can be reversed by glutamine and there is roughly a 50% inhibition of the reaction when glutamine and azaserine are present in equimolar amounts At present there are no data available for comparison of the azaserine inhibition of the PRPP glutamine reaction with the formylglycinamide ribotide reaction[6]

[5] Actually most cells in tissue culture are unable to synthesize adequate amounts of glutamine Recently Eagle *et al* (7a) have shown that mouse fibroblasts required glutamine and that HeLa cells grow optimally on glutamine but this requirement can be replaced by twenty times the amount of glutamic acid

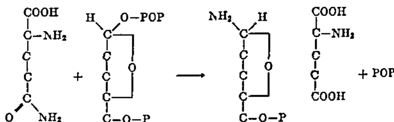
[6] Dr Buchanan in this volume presents evidence for a preferential effect on the formylglycinamide ribotide reaction

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without the acceptor system the product of the reaction is unstable and is broken down to ribose 5 phosphate This ribose 5 phosphate in turn can be converted to an organic phosphate compound which does not contain pentose and may be related to the transketolase reactions When glycine and ATP are present during the reaction of PRPP and glutamine however the product of this reaction is converted quantitatively to glycinamide ribotide In the acetone powder extract and in a sucrose

TABLE IV
INSTABILITY OF THE PRODUCT OF THE PRPP GLUTAMINE REACTION

| Preincubation | Incubation | PRPP at start of incubation μM | Glycinamide ribotide formed μM |
|---|---|---|---|
| PRPP and glutamine | Glycine and ATP | 0 | 0 |
| None | PRPP and glutamine + glycine and ATP | 0.6 | 0.3 |

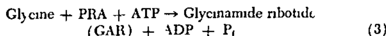
homogenate supernatant fraction the enzyme responsible for the PRPP glutamine reaction appears to be slower than the conversion of PRA to GAR This may be more than fortuitous as an excess of PRA would result in degradation to R5P and consequent loss of two ATP molecules per molecule of PRA degraded Preliminary experiments suggest that the breakdown of PRA may be enzymatic as well as nonenzymatic and this introduces another possible regulatory mechanism This degradation of PRA occurs when glycine levels are extremely low Such low levels probably do not occur normally *in vivo* as this amino acid appears to be strongly concentrated by cells (7) and is also produced from serine (1) Isotopic experiments indicate that the major source of glycine in the animal is from serine (1) Experiments also suggest that pyruvate is converted to serine and glycine by an unknown mechanism (1) Again we see a possible link with the glycolytic system and this may have some relationship to the rate of serine and glycine synthesis in normal and neoplastic tissue It is possible that extremely low levels of glycine may be produced in the animal by administration of a folic acid antagonist which will decrease the conversion of serine to glycine (9)

The final reaction sequence is the formylation of glycinamide ribotide (GAR) to yield formylglycinamide ribotide (FGAR) This requires first the formylation of a tetrahydrofolic acid derivative followed by the transfer of this formyl group to GAR

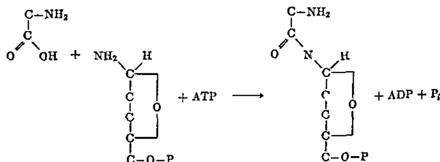
TABLE III
EFFECT OF AZASERINE ON THE PRPP GLUTAMINE REACTION

| Experiment | Glutamine μM | Azaserine μM | PRPP utilized μM |
|---|---|---|---|
| 1 | 5 | 0 | 0.29 |
| | 5 | 2.5 | 0.17 |
| | 5 | 5.0 | 0.11 |
| | 5 | 10 | 0.07 |
| | 5 | 20 | 0.06 |
| 2 | 5 | 0 | 0.14 |
| | 5 | 20 | 0.00 |
| | 10 | 20 | 0.01 |
| | 20 | 20 | 0.07 |

The second reaction between PRPP and glutamine results in a product PRA which then reacts with glycine and ATP to form glycinamide ribotide



$$\text{Glycine} + \text{PRA} + \text{ATP} \rightarrow \text{Glycinamide ribotide (GAR)} + \text{ADP} + P_i \qquad (3)$$




In this reaction a peptide bond is formed. This requires energy which is derived from ATP. Actually the mechanism of synthesis of this bond appears to be similar to the mechanism of synthesis of glutamine from glutamic acid, ammonia, and ATP.

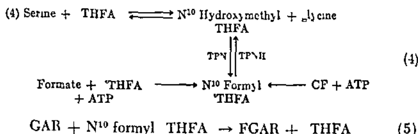
Control of the rate of this reaction again will depend on substrate levels. The level of the product of the PRPP glutamine reaction (indicated as PRA) depends on the rate of production and the rate of destruction of this compound. The latter is an important consideration at least in *in vitro* experiments and may possibly provide an internal control mechanism for synthesis of purine nucleotides. If PRPP and glutamine are preincubated for 30 minutes until the reaction is complete and then glycine-1-$C^{14}$ and ATP are added and the incubation continued for 30 minutes, no glycinamide ribotide is formed (Table IV). In other words

been isolated and it is possible that the formylation of GAR may be more sensitive

Finally it should be pointed out that the characterization of the aliphatic compound glycinamide ribotide is a point of departure for synthesis of a number of analogs possibly of the riboside series which might block the conversion of GAR to FGAR and would possess a high degree of specificity for this reaction

I wish to add a speculative note regarding the philosophy of tumor chemotherapy which I feel warrants further exploration The present objective of chemotherapy is to kill malignant cells By the bacterial analogy an antimetabolite to be most effective must block at least one metabolic pathway which is present only in the invading cell (30) No such unique pathway [with the possible exception of $B_1$ synthesis in mammary tumors (30)] has yet been demonstrated in malignant cells and the effect of an antimetabolite on a single pathway therefore lies in some more vulnerable aspect of this pathway in the tumor cell as opposed to the normal cell The reason for increased vulnerability may be due to a more favorable transport mechanism or concentrating mechanism of the antimetabolite a decreased concentration of the metabolite within the cell a less active degradation system affecting the antimetabolite an altered $K_m$ of the enzyme for the antimetabolite or possibly a different cellular locus of the enzyme system which is affected by the antimetabolite In every case the differential between the effect on normal cells and the effect on tumor cells is small In most cases the effect also depends to a major degree on the activity required of the enzyme system for example in rapidly growing tissue the normal rate of a reaction per unit of enzyme may be increased and the inhibition may be correspondingly greater For these reasons an antimetabolite designed to inhibit a pathway common to all mammalian cells usually will not kill just neoplastic cells Is it possible that some of these compounds act by altering the nature of the malignant cell?

The nature of the malignant cell must concern us in chemotherapy Malignant cells arising from different tissues resemble one another in many biological morphological and biochemical features The similarity with embryonic tissue has long been obvious and many investigators have considered neoplasia in terms of the general problem of differentiation In the process of differentiation some potentials of the cell are developed certain functions are retained and others are lost For each of these functions there are specific functional units which are involved in synthesis or maintenance of material It seems reasonable that one difference between a normal and a malignant cell is a quantita




$$\text{GAR} + N^{10}\text{ formyl THFA} \rightarrow \text{FGAR} + \text{THFA} \qquad (5)$$

The experimental work which has led to the scheme outlined under reaction 4 was done by Dr Robert Greenberg and Dr Lothar Jaenicke (15 18) Several facts are noteworthy In this scheme one carbon units are introduced at the oxidation level of formaldehyde through serine and as formate In the animal the main source of one carbon units is serine (1) and this as was mentioned previously can be synthesized from carbohydrate possibly through β OH pyruvate (8) Again the relationship of glycolysis to serine synthesis and the production of one carbon units is raised in connection with normal versus malignant cells

Another point of interest is that the one carbon unit is located on the 10 nitrogen of tetrahydrofolic acid (THFA) rather than on the 5 nitrogen as in citrovorum factor (CF) CF can be converted however to $N^{10}$ THFA by a reaction with ATP The location of the formyl and hydroxymethyl groups on the $N^{10}$ position of the folic acid derivative suggests that the $N^{10}$ methyl derivatives such as amethopterin might inhibit some of the reactions depicted *Neither amethopterin nor chemically* reduced amethopterin affects the incorporation of $C^{14}$ formate into THFA Experiments to demonstrate whether the conversion of the β carbon of serine to purines is affected have not been completed Aminopterin does not affect any of the reactions depicted but appears to inhibit the conversion of folic acid to its reduced form (3) It is not clear whether this inhibition occurs in the initial reduction to dihydrofolic acid or in the final reduction with DPN (22) to tetrahydrofolic acid The involvement of TPN in the interconversion of the hydroxymethyl to the formyl compound is of interest Finally it should be noted that "THFA" and its derivatives are represented in quotation marks as these unstable compounds may be used in model systems but are known not to be the natural cofactors

In the synthesis of the purine ring two formylation steps occur The first is the formylation of GAR (reaction 5) and the second is the formylation of 5 amino 4 imidazole carboxamide ribotide In *E coli* the latter reaction appears to be more sensitive to block by folic acid antagonists (14) In animal systems however the carboxamide has never

REFERENCES


1 Arnstein H R V Advances in Protein Chem 9 1 (1954)
2 Bernstein I A J Biol Chem 205 317 (1953)
3 Blakley R L Biochem J 58 448 (1954)
4 Buchanan J M Levenberg B Flaks J G and Gladner J A in Amino Acid Metabolism (W D McElroy and B Glass eds) p 743 Johns Hopkins Press Baltimore 1955
5 Buchanan J M Levenberg B and Lukens L N Abstr 127th Meeting Am Chem Soc (Minneapolis) p 1-C (1955)
6 Burchenal J H Federation Proc 13 760 (1954)
7 Christensen H N in Amino Acid Metabolism (W D McElroy and B Glass eds) p 63 Johns Hopkins Press Baltimore 1955
7a Eagle H Oyama V I Levy M Horton C L and Fleischman R J Biol Chem 218 607 (1956)
8 Ehrens ard G Ann Rev Biochem 24 275 (1955)
9 Elwyn D and Sprinson D J Biol Chem 184 475 (1950)
10 Goldthwait D A Greenberg G R and Peabody R A Biochim et Biophys Acta 18 148 (1955)
11 Goldthwait D A Peabody R A and Greenberg G R J Am Chem Soc 76 5258 (1954)
12 Goldthwait D A Peabody R A and Greenberg G R J Biol Chem 221 555 (1956)
13 Greenberg G R Federation Proc 10 192 (1951)
14 Greenberg G R Federation Proc 12 651 (1953)
15 Greenberg G R Jaenicke L and Silverman M Biochim et Biophys Acta 17 589 (1955)
16 Hartman S C Levenberg B and Buchanan J M J Am Chem Soc 77 501 (1955)
17 Horecker B L and Mehler A H Ann Rev Biochem 24 207 (1955)
18 Jaenicke L Biochim et Biophys Acta 17 588 (1955)
19 Kornberg A Lieberman I and Simms E S J Biol Chem 215 389 (1955)
20 Kornberg A Lieberman I and Simms E S J Biol Chem 215 417 (1955)
21 Palade G J Biophys Biochem Cytol 1 59 (1955)
22 Rauen H M and Jaenicke L Z physiol Chem 293 46 (1953)
23 Schoenheimer R The Dynamic State of Body Constituents Harvard University Press Cambridge 1942
24 Speck J F J Biol Chem 179 1387 (1949)
25 Stern J R Eggleston L V Hems R and Krebs H A Biochem J 44 410 (1949)
26 Stock C C Reilly H C Buckley S M Clarke D A and Rhoads C P Nature 173 71 (1954)
27 Strebich M J Shelton E and Schneider W C Cancer Research 13 237 (1953)
28 Waelsch H Advances in Enzymol 13 237 (1952)
29 Warburg O Science 123 309 (1956)
30 Woolley D W in Antimetabolites and Cancer" p 163 American Association for the Advancement of Science Washington D C 1955

tive difference between the number of these different functional units rather than a qualitative difference in their synthesis or structure These units are now defined in terms of subcellular structures such as nucleoli mitochondria microsomes and enzymes Further definition is obviously required The principle of a quantitative difference of functional units between normal and malignant cells is clear however It is beautifully illustrated by the work of Striebich *et al* (27) in which changes in the number of mitochondria per liver cell were induced by dimethylamino azobenzene (DAB) derivatives With 2 methyl DAB the number of mitochondria per cell increased whereas with 3 methyl DAB the number decreased If the latter compound was administered for a long enough period hepatomas developed Thus by the use of related compounds entirely opposite effects were noted on one of the functional units of the cell The same principle has recently been enunciated by Warburg in connection with the relationship of fermentation to respiration in normal and malignant cells (29) and it obviously pertains to the microsomal content as judged by electron microscopic studies of malignant tissue (21)

With the concept of the balance of subcellular units in mind it might be well to define an alternative objective in chemotherapy to the killing of cells This objective can be stated as the alteration of the metabolism of malignant cells in such a fashion that they no longer possess the outstanding property of malignant cells uncontrolled division This alteration in metabolism envisages a redistribution of the number of different units of the cell a process which takes place in normal differentiation In order to understand how to approach this objective we must define more rigorously the nature of the subcellular units in normal and malignant cells and then we must answer the question What are the factors involved in the relative rates of synthesis of these units and in the maintenance of these units? Stated in another way Why does a cell direct material principally into synthesis and maintenance of microsomes for example rather than mitochondria? Concerning the nucleic acids the same question must be raised—What controls the diversion of nucleic acids into different functional units? In ribonucleic acid metabolism there may be an initial common synthetic pathway for all units which then subdivides for specific units It is at this subdivision that a chemotherapeutic agent might act to divert nucleic acid specifically into one pathway Furthermore we should ask Do some of the agents now in use act in this manner? If we are searching for compounds which affect the process of differentiation we may have to develop better indices to distinguish between the two possible effects of chemotherapy—the death of cells and the transformation of cells

# 36

# Anticancer Agents and Nucleic Acid Metabolism of Isolated Human Leukocytes

RICHARD J WINZLER
University of Illinois College of Medicine Chicago Illinois

The preceding discussions can have left little doubt that processes of growth and cell division are intimately interwoven with biochemical reactions involving the synthesis and function of nucleic acids It was the recognition of this relationship that led us some time ago to initiate our studies on the effects of potential chemotherapeutic agents on nucleic acid metabolism of isolated human leukocytes Our approach to the problem has been to study the effects of metabolic antagonists on incorporation of labeled nucleic acid precursors into the whole cell fraction of normal and leukemic leukocytes and to determine the cell components into which these precursors are incorporated We hoped to throw some biochemical light on the problem of resistance development perhaps to provide data bearing on selection of drugs for individual cases of leukemia and perhaps also to provide a means of screening potential chemotherapeutic agents with human material We are yet a long way from any of these objectives but we do feel that we have made a measure of progress and it is of this progress that I should like to tell you The project has been made possible through a close collaboration between the biochemists of our group Dr Williams Mr Wells Miss Burr and Miss Bornstein and members of the Department of Medicine especially Drs William Best John Schoolman Louis Limarzi and John Louis I am acting as a spokesman for this group

The method of isolating the leukocytes shown in Fig 1 is essentially that of Beck and Valentine (2) making use of fibrinogen to speed sedimentation of the erythrocytes This procedure permits recovery of 60 to 80% of the leukocytes within 90 minutes after blood is drawn The

ferred to tared planchets dried weighed and the radioactivity determined From these data is calculated the micromoles of the precursor incorporated per milligram of protein

In some experiments the trichloroacetic acid soluble nucleotides have been separated by ion exchange chromatography and their specific activity determined In other experiments the RNA nucleotides and certain of the amino acids have been isolated from the "gross protein fraction" for specific activity determination

Leukocytes have proved to be very satisfactory material for biochemical studies They can be obtained in reasonable quantities with little damage to them They have an active metabolism and they can be studied in their native environment in which they continue to be active for rather long periods of time There are difficulties too Single experiments with controls and replicates require some $10^9$ cells a fact which restricts work with normal or leukopenic blood Normal cells have not proved to be a problem however because of the willingness of our medical students to bleed for the cause of science However the homogeneity of the cell populations does not compare with that achieved with the transplanted leukemias or microorganisms about which we have been hearing

In agreement with the comments during this Symposium by Drs Rouser Valentine and Waisman we conclude that leukocytes are at least as different metabolically and biochemically as they are morphologically (3) Some data demonstrating this point are shown in Fig 2 Here the in vitro incorporation of $C^{14}$ formate into different types of leukocytes is expressed as micromoles incorporated per milligram of protein in a 4 hour incubation period and plotted as a function of the formate concentration Two points of major interest are apparent In all cases formate uptake rises to a maximum at concentrations above 0 4 $\mu$M ml the uptake being very small in comparison to the amount of formate added The second point evident from these data is the large difference in the extent of formate uptake by leukocytes of different types Although these curves are for individual experiments with three or four replicate incubations for each point it has been found that the differences noted here are reproducible and consistent

Formate incorporation is two to three times as fast in Krebs Ringer bicarbonate glucose solution as it is in serum The cells frequently clumped in this and in other media however We have therefore preferred to maintain the cells in as physiological an environment as possible by suspending them in normal serum

preparations are not completely free of erythrocytes and often there may be equal numbers of erythrocytes and leukocytes in the final preparations This contamination has not been a serious problem as yet however since control experiments with erythrocytes alone have shown negligible isotope uptake with the precursors we have used

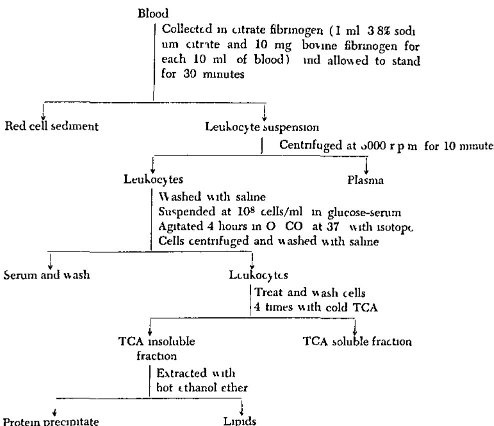



FIG 1 Isolation and incubation of leukocytes

This figure also shows the procedure used in most of the incubations and in the subsequent fractionation procedures One hundred million cells are incubated in 2 2 ml of normal serum containing 2 $\mu c$ of isotopic precursor in the presence and absence of the inhibitor under study At the end of the incubation period the cells are harvested washed the proteins precipitated with trichloroacetic acid and the lipids extracted to yield the "gross protein fraction with which much of our work has been concerned This protein is then dissolved in formic acid trans

ferred to tared planchets dried weighed and the radioactivity determined From these data is calculated the micromoles of the precursor incorporated per milligram of protein

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Formate uptake is linear with time over at least a 6 hour period providing sufficient glucose is added This is shown in Fig 3 for cells from a patient with chronic granulocytic leukemia Also evident is the fact that formate incorporation into the gross protein fraction is markedly

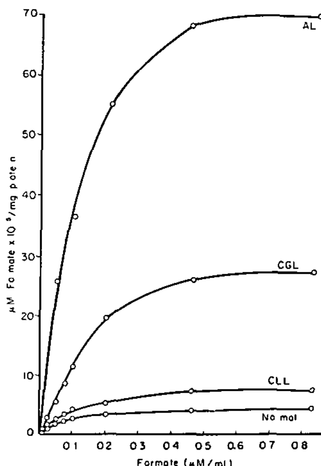



Fig 2 Formate incorporation into the "gross protein fraction" of leukocytes as a function of formate concentration AL = acute leukemia cells CGL = chronic granulocytic leukemia cells CLL = chronic lymphocytic leukemia cells Normal = normal cells

inhibited by amethopterin Since the amethopterin was added to the incubation vessels just before addition of radioformate it is evident that the effect of amethopterin is immediate and nonprogressive This suggests that there is no great permeability barrier or extensive change of the antimetabolite required before it exerts its metabolic effect

The amethopterin concentration in this experiment was very high 100 γ/ml Since the incorporation of formate was not completely inhibited by this high concentration it appears that there may be an amethopterin resistant formate incorporation into these leukocytes Figure 4 presents further data bearing on this point Here again are seen the differences in the rates of formate incorporation by the different types of leukocytes The different types also show striking differences

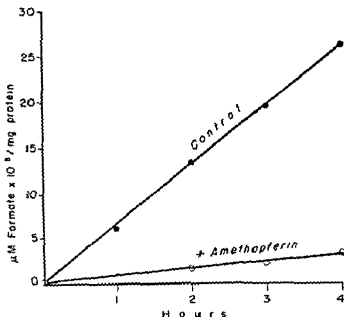



Fig 3 Formate incorporation by chronic granulocytic leukemia cells as a function of time Effect of amethopterin

in sensitivity to amethopterin Formate uptake by cells from normal individuals or from patients with chronic lymphocytic leukemia was essentially unaffected by amethopterin at any concentration Formate incorporation by cells from patients with granulocytic leukemia was markedly inhibited usually by 80% or more whereas that of cells from patients with adult acute leukemia was inhibited by only 30 to 50%

Several interesting questions may be raised at this point Can clinical resistance to the folic acid antagonists be related to differences in relative extents of the resistant and sensitive pathways of formate metabolism as clinical resistance to the drug develops? Is the partial resistance of

the granulocytic cells by amethopterin due to the presence of two populations of leukocytes one sensitive to and the other resistant to amethopterin or are there two pathways of formate incorporation in all the cells? These are some of the questions on which we have speculated and which we hope to answer in future work

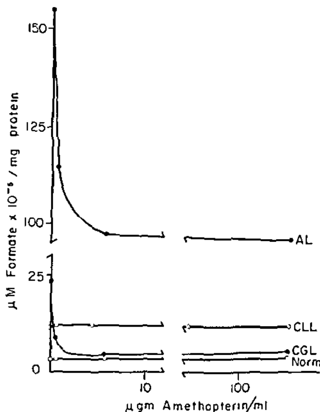



FIG 4 Formate incorporation by leukocytes as a function of amethopterin concentration Abbreviations same as in Fig 2

If the effect of amethopterin is a specific one it should be reversible by citrovorum factor This is in fact the case as is shown in Fig 5 Two points are evident—the addition of leucovorin causes a stimulation of formate incorporation into the CGL cells and the inhibitory effect of amethopterin can be overcome by relatively large amounts of leucovorin The relation is such as to suggest a competitive relationship between amethopterin and citrovorum factor Folic acid exerts neither of these effects

The effects of a number of other inhibitors on formate incorporation have also been studied Unfortunately there are several complications in the use of some of these inhibitors in comparison with amethopterin In the first place the dose response curves are far less consistent and inhibition may require far larger concentrations—outside those which

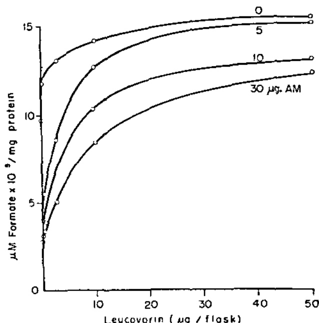



FIG 5 Reversal of amethopterin inhibition of formate incorporation by leucovorin

could conceivably be involved in clinical chemotherapy—for *in vitro* effects Some of these data are shown in Fig 6

Another problem is the fact that some inhibitors may show a considerable time lag before inhibition becomes manifest Even high concentrations of azaserine for example do not inhibit at all for the first 30 to 60 minutes and maximum effectiveness is achieved only after 2 to 3 hours It is evident that consistent results with azaserine will require a preincubation period since the extent of inhibition depends on how long the incubation is run In spite of these difficulties which tend to obscure rational interpretation I have summarized in Table I the effects of a number of inhibitors on the incorporation of formate into the "gross protein fraction" At the top of this table the means and standard deviations of formate uptake into the four types of leukocytes are expressed as micro

the granulocytic cells by amethopterin due to the presence of two populations of leukocytes one sensitive to and the other resistant to amethopterin or are there two pathways of formate incorporation in all the cells? These are some of the questions on which we have speculated and which we hope to answer in future work

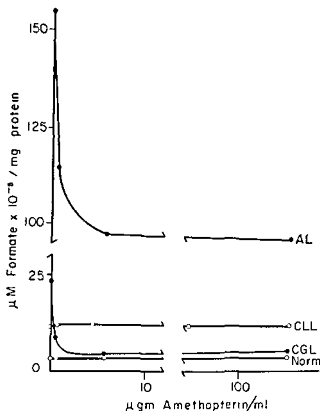



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moles $\times 10^5$ per milligram of protein in a 4 hour incubation period As has already been pointed out only the CGL cells are highly sensitive to amethopterin with the acute leukemia cells being moderately inhibited and the normal and CLL cells being essentially unaffected. So far as it has been studied the effects of Daraprim mimicked the effects of amethopterin

Although Myleran has a marked effect on the clinical course of chronic granulocytic leukemia it does not affect the *in vitro* incorporation of formate into the leukocytes in this *in vitro* system Neither does it affect

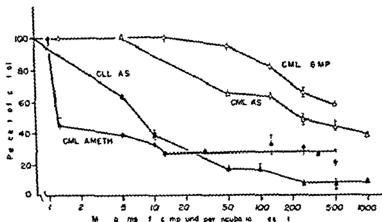



FIG 6 Dose-response curves for some inhibitors of formate incorporation CGL = chronic granulocytic leukemia CLL = chronic lymphocytic leukemia AMETH = amethopterin AS = azaserine 6MP = 6-mercaptopurine

the incorporation of other isotopic precursors that have been used including *glycine adenine and phosphate*

6-Mercaptopurine has shown only moderate effects on formate uptake even at very high concentrations

Azaserine is uniformly inhibitory on formate uptake into all types of abnormal leukocytes and somewhat less so with the normal cells Some of the difficulties of interpretation of these data have been mentioned however

Cortisone has had no effect on formate uptake in any of the cells investigated

Studies on the effect of these agents on the incorporation of other isotopic precursors into the gross protein and *nucleic acid fraction have* been too limited to merit such credence With this reservation however

TABLE I

EFFECT OF SEVERAL AGENTS ON FORMATE INCORPORATION INTO THE GROSS PROTEIN FRACTION OF LEUKOCYTES

| Inhibitor | Formate uptake in Normal cells 4.8 ± 0.6 (33) | CLL cells 11.1 ± 5.1 (35) | CGL cells 22.1 ± 8.2 (49) | AL cells 60.6 ± 15.2 (13) |
|---|---|---|---|---|
| | Per cent inhibition | | | |
| Amethopterin (100 γ/ml) | 3.9 ± 4.1 (33) | 6.7 ± 5.4 (25) | 60.5 ± 8.2 (49) | 35.0 ± 4.5 (13) |
| Daraprim (100 γ/ml) | — | − 8.0 ( 3) | 83.8 ± 6.8 ( 8) | — |
| Myleran (100 γ/ml) | — | − 2.9 ± 15.2 ( 5) | − 0.6 ± 8.1 (13) | − 6.0 ( 1) |
| 6-Mercaptopurine (100 γ/ml) | 27.5 ± 10.1 ( 5) | 0 ± 9.5 ( 8) | 32.0 ± 23.1 (17) | 1.7 ± 19.1 ( 9) |
| Azaserine (450 γ/ml) | 22.1 ± 8.7 ( 4) | 56.1 ± 15.2 (14) | 59.2 ± 5.5 ( 5) | 58.2 ( 2) |
| Cortisone (100 γ/ml) | − 4.5 ± 10.5 ( 4) | − 2.8 ( 3) | 6.5 ± 16.0 ( 6) | −16.1 ± 1.3 ( 4) |
| 2,6-Diaminopurine (100 γ/ml) | — | — | 40.7 ± 6.2 ( 5) | — |

Expressed as micromoles of formate × $10^5$ incorporated per milligram of protein in 4 hours at 37° in an atmosphere of 95% $O_2$–5% $CO_2$ and a medium of normal serum containing 6 mg of glucose and 0.67 μM of formate per milliliter with standard deviation. The number of experiments is given in parentheses.

moles × 10 per milligram of protein in a 4 hour incubation period As has already been pointed out only the CGL cells are highly sensitive to amethopterin with the acute leukemia cells being moderately inhibited and the normal and CLL cells being essentially unaffected So far as it has been studied the effects of Daraprim mimicked the effects of amethopterin

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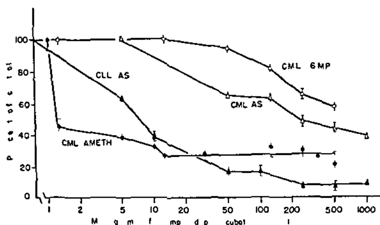



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Studies on the effect of these agents on the incorporation of other isotopic precursors into the gross protein and nucleic acid fraction have been too limited to merit such credence With this reservation however

I should like to indicate the status of this work at the present time Table II shows the effects of several agents on the incorporation of glycine 1 $C^{14}$ into the gross protein fraction of the different types of leukocytes each value representing the average of two or three separate experiments It is seen that the rate of glycine incorporation into leukocytes follows the same order as that obtained with formate Amethopterin had no effect on glycine incorporation of any of the cells Isolated RNA nucleotides or amino acids have not yet been studied in cells incubated with $C^{14}$ glycine and any differential effects into these components are not yet evident However most of the radioactivity with this isotope no doubt resides in the amino acids—primarily serine and glycine

TABLE II
EFFECT OF SEVERAL AGENTS ON GLYCINE 1 $C^{14}$ INCORPORATION INTO THE PROTEIN FRACTION OF LEUKOCYTES

| Inhibitor | Glycine 1 $C^{14}$ uptake in | | |
|---|---|---|---|
| | Normal cells 30 4 | CLL cells 84 5 | CGL cells 310 |
| | Per cent inhibition | | |
| Amethopterin (100 γ/ml ) | 2 5 | 3 0 | 3 8 |
| 6 Mercaptopurine (100 γ/ml ) | — | 0 | — |
| Myleran (100 γ/ml ) | — | — 4 0 | 4 0 |
| Azaserine (300 γ/ml ) | 27 | 78 | 26 |

Expressed as micromoles × 10 incorporated per milligram in 4 hours

Myleran also had no effect on glycine incorporation into the gross protein fraction The only agent which had any consistent effect was azaserine with a suggestion that CLL cells are somewhat more sensitive than CGL or normal cells Results somewhat parallel with those obtained with $C^{14}$ glycine have been obtained in preliminary experiments with cells incubated with adenine 8 $C^{14}$ The order of isotope uptake is the same as that observed with formate and glycine The most pronounced inhibitory effect was obtained with azaserine acting on cells from patients with chronic lymphocytic leukemia These results with glycine and adenine must be regarded as highly tentative since they represent experiments with only two or three patients

Thus far we have talked only about the incorporation into the gross

protein fraction For any real insight into leukocyte metabolism and the action of antileukemic agents it is essential to know more about the components and the positions into which isotopic precursors are incorporated Formate for example is incorporated into the purines adenine and guanine and into serine methionine and histidine Does incorporation into all these components involve a common intermediate and do agents like amethopterin inhibit equally the incorporation into all these components? Figure 7 serves as a point of departure for consideration

TABLE III

Effects of Inhibitors on Adenine 8 $C^{14}$ Incorporation into the Protein Fraction of Leukocytes

| | Adenine 8 $C^{14}$ uptake in | | |
|---|---|---|---|
| | Normal cells 34 | CLL cells 63 | CGL cells 73 |
| Inhibitor | Per cent inhibition | | |
| Amethopterin (100 γ/ml ) | 35 | 26 | 16 |
| 6 Mercaptopurine (100 γ/ml ) | – | 12 | 6 |
| Myleran (100 γ/ml ) | – | 12 | 15 |
| Azaserine (300 γ/ml ) | 13 | 81 | 9 |

Expressed as micromoles × $10^{5}$ incorporated per milligram in 4 hours

of this question and suggests two situations for formate incorporation by alternate pathways

Consider the case where amethopterin inhibits only the upper pathway of each situation leaving the lower pathways unaffected In the upper situation the distribution of the isotope in the final products would be unaltered In the lower situation however the incorporation of the isotope into certain products would be markedly reduced whereas the incorporation into others would not be markedly affected

Experimentally the effect of amethopterin on formate incorporation corresponds to the upper situation namely a parallel inhibition of incorporation into all the components studied This is shown in Table IV Here it is evident that amethopterin inhibits the incorporation of radio formate into the total protein fraction by about 80% The incorporation into RNA adenine RNA guanine protein bound serine and acid soluble

TABLE IV

Distribution of Radioactivity from C[14] Formate in Several Components of CML Cells Effect of Amethopterin

| Gross protein | Control 4 1 × 10 μc /100 mg protein | | Amethopterin inlubited 0 9 × 10 μc 100 mg protein | |
|---|---|---|---|---|
| | μc /μM | μc /100 mg protein | μc /μM | μc /100 mg protein |
| I rotein bound serine | 0 12 × 10 | 2 9 × 10 | 0 02 × 10 | 0 58 × 10 |
| RNA adenine | 0 13 × 10 | 0 1 × 10 † | 0 037 × 10 | 0 028 × 10 † |
| RNA guanine | 0 1 × 10 | 0 17 × 10 ‡ | 0 01 × 10 | 0 02 × 10 ‡ |
| Acid soluble ATI | 1 0 × 10 | – | 0 09 × 10 | – |

Calculated from average of 2 5% serine in gross protein fraction

† Calculated from an estimated content of 0 78 μM RNA adenine per 100 mg protein

‡ Calculated from an estimated content of 1 72 μM RNA guanine per 100 mg protein

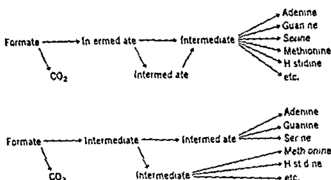



Fig 7 Possible alternate pathways of formate metabolism

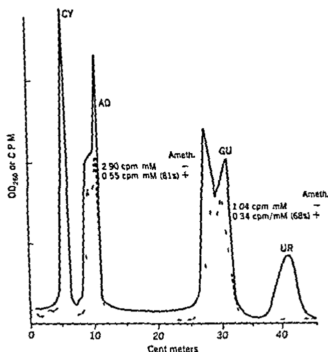



Fig 8 Incorporation of $C^{14}$ formate into the purines of ribonucleic acid of CGL cells RNA isolated after incubation of CGL cells with $C^{14}$ formate was hydrolyzed to the nucleotides These were separated by chromatographic methods from radioactive contaminants and the nucleotides separated by paper ionophoresis One-centimeter segments were eluted and the concentration and radioactivity of each eluate determined

ATP (and other acid soluble nucleotides) was inhibited to an equal extent It is evident from this work that most of the radioactivity in the protein bound fraction resides in serine and less than 10% in the RNA purine nucleotides This table also shows that the specific activity of the acid soluble ATP was some tenfold higher than the specific activity of the RNA nucleotides

Figure 8 shows the results of ionophoretic separation of the nucleotides from RNA of chronic granulocytic cells incubated with $C^{14}$ formate

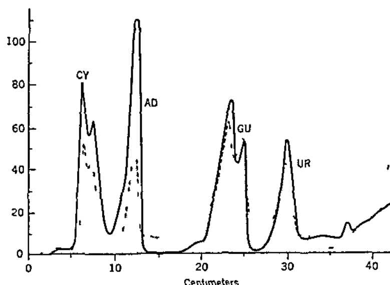



FIG 9 Incorporation of $P^{32}O_4$ into the nucleotide of ribonucleic acid of CGL cells RNA isolated after incubation of CGL cells with $P^{32}O_4$ was hydrolyzed to the nucleotide The e were separated by chromatographic methods from radioactive contaminants and the nucleotides separated by paper ionophoresis One centimeter segments were eluted and the concentration and radioactivity of each eluate determined

| Fraction | RSA control | RSA amethopterin |
|---|---|---|
| Protein bound P | 1 9 | 1 9 |
| Cytidylic P | 2 6 | 2 5 |
| Adenylic P | 4 6 | 4 6 |
| Guanylic P | 3 4 | 3 1 |
| Uridylic P | 4 3 | 4 9 |

$$\text{RSA} = \frac{\text{c p m}/\mu\text{M P in fraction} \times 100}{\text{c p m}/\mu\text{M P in inorganic} \times 100}$$

It is seen that only the adenine and guanine have radioactivity and that *the activity of both is decreased to 20 to 30% of the uninhibited level by* the presence of amethopterin

The situation observed when the incubation is carried out with radio active phosphate instead of with $C^{14}$ formate is quite different This is shown in Fig 9 Here all the RNA nucleotides are radioactive as ex pected The presence of amethopterin had no effect on the specific activities of the nucleotides however This dissociation of $P^{32}O_4$ from $C^{14}$ formate turnover demonstrates that incorporation of phosphate and of formate may proceed independently and may indicate that such in corporation may not represent true *de novo* synthesis of ribonucleotides

In summary then it is evident that human leukocytes of different types demonstrate interesting differences in metabolism They differ markedly in their rates of nucleic acid and protein biosynthesis or turn over and in their sensitivity to chemotherapeutic agents Such observa tions hold forth some hope that these differences may one day be ex ploited *in the chemotherapy of leukemia The data are still too frag* mentary to permit any conclusions correlating clinical with *in vitro* effectiveness of any drug If such a correlation for any agent is found however we may have a means of selecting on a rational basis the drug to be used with individual leukemia patients It is possible that resistance to these agents may be detected biochemically before clinical resistance becomes manifest In any case it will be most in teresting to see whether the mechanism of action of potential chemo therapeutic agents can be elucidated whether the biochemical basis for resistance can be determined and whether this method can be used to screen potential chemotherapeutic agents on human material

REFERENCES

1 Beck W S *J Biol Chem* 216 333 (1955)
2 Beck W S and Valentine W N *Cancer Research* 12 818 (1952)
3 Valentine W N *Blood* 6 845 (1951)
4 *Weisberger A S and Levine B Blood 9 1082 (1954)*

# 37

## Studies on Resistance to Folic Acid Antagonists[1]

CHARLES A NICHOL
Department of Pharmacology School of Medicine
Yale University New Haven Connecticut

The importance of drug resistance as a serious limitation on the usefulness of compounds employed in the treatment of bacterial or parasitic infections of man or used in the control of insect vectors of disease is well known (12 28 38 39 53 80 94) Even more important is the problem of drug resistance in the chemotherapy of neoplastic diseases thus in leukemia defense mechanisms seem to be relatively ineffective against aberrant cells produced by the body In some cases at least the effective chemotherapy of leukemia may require the destruction of all neoplastic cells The experiments of Furth and Kahn (41) which showed that a lymphoid leukemia in mice can be transferred by means of a single leukemic cell give emphasis to this important aspect of the problem A similar qualification for effectiveness may be applicable to the chemotherapy of solid tumors as more effective agents become available for their treatment since drug resistance has been observed in experimental neoplasms other than leukemia for example in sarcoma 180 to 6 mercaptopurine (23) and in the Walker rat sarcoma to a derivative of nitrogen mustard (24)

The eventual failure in the continued usefulness of those drugs which are effective initially in the treatment of certain leukemic individuals is generally attributed to the emergence of a drug resistant population of leukemic cells *although direct proof of this postulate is lacking* The considerable extrapolation from studies of resistance in transplantable leukemias in mice to resistance in the spontaneous leukemias of humans

[1] These studies were supported in part by a research grant from the National Institutes of Health United States Public Health Service

Present address Roswell Park Memorial Institute Buffalo New York

should be recognized A logical strategy requires exploration of the problem in the area where such studies are technically feasible how ever with the intent that the characteristics which can be associated with drug resistance in mouse leukemias may eventually be tested by the direct study of human leukemic cells to provide an explanation for the failures in treatment after complete remissions The development of drug resistant lines of leukemia in mice (18 67 69 76 77) and their detailed study (73) has broadened our perspective of the problem and certain correlations between mouse and human leukemias provide an encouraging basis for such investigations (16 74)

Law and co workers (77) have presented a sobering observation namely that resistance to 6 mercaptopurine in leukemic mice (L 1210) unlike bacterial resistance to this compound is accompanied by resist ance to all other purine analogs tested 8 azaguanine 8 azaxanthine 2 6 diaminopurine thioguanine purine and chloropurine In contrast a 6 mercaptopurine resistant strain of *L casei* remained sensitive to ade nine antagonists (33) The occurrence of cross resistance among the folic acid antagonists which was noted earlier in different mouse leu kemias (21 69 72) has been observed clinically Murphy and co workers (81) found that the development of resistance to amethopterin (Methotrexate) in children with acute leukemia conferred resistance to other folic acid antagonists including the diaminodichlorophenylpyrim idines but not to 6 mercaptopurine or cortisone Although the number of new effective agents may increase rapidly the number of potentially useful new antileukemic compounds is likely to be limited appreciably by the occurrence of cross resistance However cross resistance may provide an important indication of similarities in the mechanism of action of different antimetabolites or of similarities in the means by which variant cells are able to circumvent the adverse effects of related drugs

In addition to resistance a phenomenon described as dependence" has been observed in some sublines of leukemia (67 68 77) The ad ministration of certain antagonists of folic acid or purine analogs to leukemic mice carrying the respective sublines caused significant ac celeration of the rate of growth of leukemic tissues resulting in a de crease in the survival time of the treated animals as compared with the untreated mice Instances of microbial dependence on various antibiotics also have been described (39) Miller and Bohnhoff (78) found that meningococcal variants dependent on streptomycin remained nonviru lent for mice unless the animals received streptomycin in which case a fatal meningococcal sepsis developed The direct study of the metabolism of amethopterin dependent leukemic cells demonstrated that the presence

of amethopterin actually doubled the rate of synthesis *de novo* of nucleic acids as measured by the incorporation of sodium formate $C^{14}$ (98) Furthermore a reversal of the metabolite antimetabolite relationship was demonstrated in an amethopterin dependent leukemia in which pteroyl glutamic acid (synthetic folic acid) or leucovorin (synthetic folinic acid) counteracted the growth promoting activity of amethopterin (67) A similar paradox was found in the reversal by guanylic acid and guanosine of the growth promoting activity of 8 azaguanine for the 8 azaguanine dependent leukemic cells (68 73) At present there is no satisfactory explanation of this baffling phenomenon

*The occurrence of resistance of leukemic cells to one drug (or a group* of related compounds) may be associated with changes in the metabolism of the cells in a way that confers sensitivity to a different type of compound A most significant finding by Law and co workers (74 77) which may lighten the shadow of cross resistance concerns an increased sensitivity to amethopterin associated with resistance to or dependence on 6-mercaptopurine or 8-azaguanine However other amethopterin resistant or dependent leukemias derived from this line (L-1210) were not more sensitive than the parent line to the purine antagonists Burchenal (17) has noted an increase in sensitivity to 6 mercaptopurine in an amethopterin resistant line (I/A) of mouse leukemia The term "collateral sensitivity has been used in microbial studies to designate the increased sensitivity to one antibiotic associated with increased resistance to another (100) Although this phenomenon has been noted infrequently with antibiotics (99 100) several observers have described the increased sensitivity to amethopterin of selected bacterial strains which were resistant to 6-mercaptopurine (32 35) and the increased sensitivity to purine and pyrimidine analogs of amethopterin resistant and sulfonamide resistant bacteria (50 54 93) The occurrence of collateral sensitivity would seem to imply the existence of an interrelationship *between the metabolic pathways affected by the two types of* antimetabolites

In an important extension of earlier studies Law (75) has found that the treatment of a lymphocytic leukemia of mice with amethopterin after a brief course of therapy with 6-mercaptopurine resulted in a very much greater antileukemic activity than could be achieved by the simultaneous administration of these two agents or by a reversal of this sequence of treatment Although in other experiments as has been described an increased sensitivity to amethopterin was associated with resistance to the purine antagonists it is doubtful that these results could be attributed to the selection of a population of cells resistant to

6 mercaptopurine In other experiments leukemic lines resistant to 6 mercaptopurine or amethopterin were selected only after repeated transfers of the cells in animals receiving the antagonists (72 77) Also leukemic cells transferred from moribund animals which had received an initial course of therapy with either agent alone appear to retain their sensitivity to the drugs (75) Clearly the changes in metabolism associated with collateral sensitivity and with the increased effectiveness of sequential treatments deserve intensive study These investigations imply that the metabolism of the leukemic cells can be manipulated by appropriate treatment of the host with chemical agents in a manner that may amplify the meager basis for the selective toxicity of the presently available antileukemic drugs

## The Origin of Drug Resistance

Although we are concerned more with the characteristics of resistant cells in this presentation than with the origin of these traits several comments in this latter regard may be pertinent The development of our ideas concerning the origin of resistant leukemic cells has relied on important advances in the field of microbial genetics (12 22) Certain instances of resistance to antibiotics such as streptomycin resistance in *E coli* can be attributed to gene mutations which can occur in the *absence of the drug* (12 22 82) *Adaptive processes or drug induced* resistance appear to be of lesser importance in microbial resistance than the stable heritable resistance associated with spontaneous mutations (12) although an adaptive phenomenon can occur in certain instances (26) Hotchkiss (58 59) has shown that purified preparations of deoxyribonucleic acid derived from resistant variants can transmit the property of resistance to penicillin or streptomycin in pneumococci In each case transformations to high or low levels of resistance could be related to the level of resistance of the strain used for the preparation of the transforming extract (59) Also linked characteristics were transferred by DNA from strains bearing two mutant properties (60) This process apparently *involves the transfer of a mutation by means of the isolated* genetic material indicating that the deoxyribonucleic acid of the resistant cell is indeed woven in a pattern differing from that of the sensitive cell It is not clear whether a similar phenomenon was involved in the *restoration of sensitivity* to penicillin of penicillin resistant bacteria exposed to sensitive cultures or to bacterial autolyzates (43 102 106) These studies raise a question as yet unanswered as to whether a corresponding transforming principle can be associated with drug resistance in leukemic cells

Resistance to amethopterin in mouse leukemias has been shown to be a stable heritable characteristic which persists even after repeated transfer of the cells in the absence of the drug (14, 69, 74). Law (71) applied the experimental design of the Luria-Delbruck fluctuation test to a study of amethopterin resistance in an experimental leukemia (L 1210). This elaborate experiment provides the best evidence available that resistance arises spontaneously in the leukemic cells and that the drug, as in microbial resistance to antibiotics, acts solely as a selecting agent. Further, resistance to amethopterin in this leukemia increased in a stepwise manner on successive transfers (71, 72), a situation analogous to the multistep pattern of resistance to penicillin in bacteria (11, 27). The occurrence of another pattern of resistance in a plasma cell leukemia was observed by Potter (75, 92), who found that profound resistance to azaserine occurred after a single transfer. This rapid development of resistance appears to be a counterpart of the one-step microbial resistance observed with streptomycin (27) and other drugs (101). Such an occurrence would place a serious limitation on the effectiveness of an antileukemic agent.

The evidence that drug resistance in experimental leukemias is mutational in origin provides a rational basis for an attempt to suppress the emergence of a resistant population by the combination of two or more drugs with differing actions, in order to limit the opportunity for the occurrence of two or more mutations conferring resistance to each drug. Combinations of amethopterin with certain purine analogs have been found to be measurably more effective in experimental leukemias than either drug alone (70, 77, 96), although the degree of potentiation attained has not been impressive; similar combinations are receiving clinical evaluation. It is not clear to what extent such synergism may result from suppression of the development of otherwise resistant strains or from an increased potency of the combinations as compared to either drug alone. The mutational origin of resistance would also require increased attention to any mutagenic property of a new drug. The possibility that a drug may suppress the emergence of resistant populations of cells by slowing the rate of mutation appears to be impractical but not impossible. Thus the nucleoside guanosine reduced the spontaneous rate of mutation to T5 phage resistance in *E. coli* (91). An intriguing effect on the emergence of a resistant population relates to the observation that the presence of a very small amount of uracil in the medium in which *S. faecalis* is grown prevented the emergence of mutants resistant to 6-azauracil, a selection of which occurs invariably in the absence of the metabolite (51).

The selective action of amethopterin (or other drugs) on leukemic cells as opposed to normal tissues must vary considerably in different individuals Thus in the acute lymphocytic leukemia of children the effectiveness of amethopterin covers a spectrum from complete remission to little or no beneficial effect (15 36) In the body a barrier which presumably limits the degree of antileukemic action attainable with this drug is the severely toxic effect which it exerts at higher dosage levels on normal cells particularly those of the hematopoietic tissue and the intestinal epithelium (In the test tube strains of bacteria can be selected which are resistant to concentrations of amethopterin or other drugs limited only by the solubility of the compound in the culture

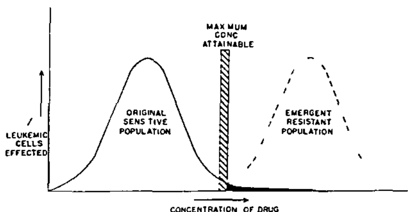



FIG 1 The emergence of a drug resistant population of leukemic cell

medium ) The proportion of the population of leukemic cells susceptible to increasing concentrations of a drug in any individual undoubtedly follows some normal distribution curve (Fig 1) The barrier to higher dosage imposed by the susceptibility of normal cells indicated as "maximum concentration attainable" may occur at the average sensitivity of the leukemic population (no beneficial effect) in one individual whereas in another such a barrier may occur at a concentration permitting the survival of relatively few leukemic cells (good remission) as indicated by the darkened area in Fig 1 The growth of those cells would give rise in time to a population capable of surviving in slightly higher concentrations of the drug From the standpoint of practical chemotherapy in order to be described as "resistant" the emergent population of leukemic cells needs only to possess a degree of sensitivity to the drug analogous to that of certain normal tissues Thus slight changes in certain characteristics of the leukemic cell rather than the

occurrence of striking differences may be sufficient to confer chemotherapeutic resistance and may be of major importance in the origin of the resistant cells in the human leukemias

#### The Nature of Drug Resistance

A question to which we need an answer concerns the characteristics of the resistant cells *directly responsible* for the loss of sensitivity to amethopterin or other agents Such an answer requires an evaluation of the different characteristics which may be involved The various mechanisms that may account for the resistance of leukemic cells are suggested by a broad survey of different instances of drug resistance in bacteria and microorganisms (3 25 28 29 39 55 57 72 89 97) these may be listed as follows

1 Inactivation of the drug or decreased activation
2 Reduced uptake of the drug associated with an altered permeability or a reduced binding capacity
3 Ability to bind the drug in an inactive form
4 Increased formation of a competing metabolite
5 Increase in the amount or the efficiency of the enzyme using the metabolite
6 Decrease in the relative affinity of the susceptible enzyme for the chemotherapeutic agent as compared with the affinity for the appropriate metabolite
7 Development of an alternate metabolic pathway
8 Utilization of exogenous metabolites which are products of the inhibited reactions

Two general observations can be made concerning these different means by which a cell may circumvent the toxic action of a drug only a few logical possibilities are apparent at present and most of these can be tested experimentally In many cases however the lack of a detailed understanding of the site of action of the drug limits an approach to the problem In any single instance of drug resistance an evaluation of the relative importance of any individual characteristic requires investigation of the possible occurrence of these characteristics

#### A Model System for the Study of Drug Resistance

Resistance to amethopterin has several advantages as a model which permits more detailed study than is possible with other antileukemic agents or antibiotics (1) the metabolic alteration of folic acid by leukemic cells *in vitro* is inhibited by concentrations of amethopterin which are attainable in plasma and tissues (19 40 83) (2) metabolic systems activated by the functional forms of folic acid such as certain

steps in the biosynthesis of purines (13 46 49) or the interconversion of serine and glycine (7 8 63 66) are suitable for the comparative study of these reactions in sensitive and resistant cells (3) methods are available for the separation (ion exchange and paper chromatography) and determination (sensitive microbial fluorometric or colorimetric assays) of both the antagonist and the products of the reactions involved (6 108) (4) amethopterin resistant strains of bacteria (20 56) and amethopterin resistant and dependent sublines of leukemia in mice (18 67 69 76) have been developed (5) the phenomena of cross resistance and collateral sensitivity can be studied and (6) the direct study of cells from leukemic patients both sensitive to or initially or subsequently refractory to treatment with amethopterin is feasible

A detailed study of resistance to the folic acid antagonists may be useful in suggesting characteristics which may be applicable to instances of resistance to other drugs However the nature of resistance to an analog of a vitamin which interferes with the formation or function of a cofactor may differ considerably from resistance to an analog of a component of nucleic acid which may interfere with the incorporation of a structural unit into nucleic acids or exert its effect by the formation of fraudulent nucleic acids The extent to which analogs of purines and pyrimidines can interfere with the formation or function of cofactors containing their corresponding metabolites has not yet been determined but catalytic functions would be expected to be most sensitive in reflecting measurable effects resulting from such inhibition

## The Site of Action of the 4-Amino Analogs of Folic Acid

The effectiveness of aminopterin and amethopterin in blocking the conversion of pteroylglutamic acid (PGA) to folinic acid (citrovorum factor) *in vitro* and *in vivo* (88) has been studied extensively (6 105) More recently we have found that the alteration of PGA to $N^5$ formyltetrahydro PGA (folinic acid SF leucovorin) can be divided into two steps an enzymatic reaction and one which can occur nonenzymatically (*1*) the metabolic product derived from PGA on incubation with preparations of pigeon liver or suspensions of bacterial or leukemic cells appears to be a heat labile compound (2) this product can be converted to the stable N formyltetrahydro PGA on heating in the presence of ascorbic acid or related compounds (under aerobic conditions the labile compound appears to be oxidized to a product presumably $N^{10}$ formyl PGA which is measurable as folic acid but which does not have folinic acid activity) (84 86 89 109) The first step but not the second is inhibited by the 4 amino analogs of PGA

The apparent specificity of the requirement of the organism used for the citrovorum factor or folinic acid assay (*Pediococcus cerevisiae* 8081 better known as *Leuconostoc citrovorum*) is related to the unique stability of the $N^5$ formyl derivative of tetrahydro PGA during the auto claving of the diluted samples with the medium in the usual microbial assay The folinic acid activity of other reduced derivatives of PGA is measurable by the addition of unheated samples protected by the presence of sodium ascorbate to assay tubes containing autoclaved medium (84) or by a pad plate assay (64) in which the samples on filter paper disks are placed on solid medium containing ascorbate (84) In order to measure accurately the product of the reaction inhibited by amethopterin it is necessary to use a modified procedure for the assay or to autoclave the incubated material at 120 for 30 minutes in the presence of ascorbate to ensure conversion of the heat labile product to the stable form of folinic acid thus permitting an indirect measure ment of the product by the use of the usual microbial assay We have found that the latter procedure is more convenient when it is necessary to assay a large number of samples

The effect of aminopterin in blocking the formation of the heat labile compound by leukemic cells is shown by the tracings of the folinic acid bioautograms (Fig 2) the main product derived from PGA was a heat labile compound at $R_f$ 0 2 which was converted on heating to a compound behaving analogously to synthetic folinic acid ($R_f$ 0 6) In cubations carried out in the presence of aminopterin resulted in the appearance of only faint zones of growth of the amethopterin resistant organism The addition of aminopterin after the incubation as expected did not influence the formation of the stable folinic acid from the heat labile compound A similar labile compound appeared in the urine of normal subjects after the ingestion of PGA (84 110) Recent reports suggest that this compound is similar to anhydroleucovorin (1 95)

Blakley (7 8) demonstrated that aminopterin blocked the ability of PGA but not tetrahydro PGA to activate the interconversion of serine and glycine These investigations indicate that the 4 amino analogs of PGA interfere with the reduction of the pteridine ring necessary for the formation of the functional form of folic acid certainly they interfere less effectively with the utilization of synthetic folinic acid which is also altered in the body to the functional forms of the vitamin (9) Since large amounts of folinic acid can counteract the toxic action of these antagonists Goldin and co workers (45) have tested the possibility that a selective protective effect of folinic acid for normal tissues might per

mit the use of larger doses of these antagonists necessary to achieve greater antileukemic action

### A Test for the Sensitivity of Leukemic Cells to Amethopterin

Suspensions of ascitic cells were much more satisfactory than preparations of lymphomatous tissue of the same line of leukemia (L 4946) for the study of the effect of amethopterin on the conversion of PGA to products measurable as folinic acid (87) A modified Krebs Ringer

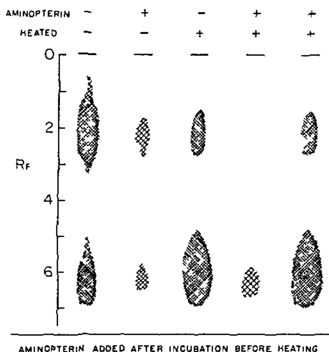



FIG 2 Tracings of folinic acid bioautograms showing the inhibition by aminopterin of the formation by leukemic cells (L 4946) of a heat labile compound derived from pteroylglutamic acid Suspensions of ascitic cells (L 4946) were incubated with PGA under the conditions described in Table I Aminopterin (1 0 γ/ml) was added to some of the vessels as indicated At the end of the anaerobic incubation the content of each vessel was covered with toluene frozen and thawed five times and then centrifuged The supernatant fluid of some of the samples was autoclaved at 120 for 20 minutes Portions (0 05 ml) of each sample were placed on paper strips (Whatman No 3 1 inch in width) which were developed at 4 by descending solvent (0 1 M phosphate buffer pH 6) The paper strips were placed on solid assay medium (folinic acid) containing sodium ascorbate (1 mg/ml) which was seeded with an aminopterin resistant strain of *Pediococcus cerevisiae* Zones of growth indicate the location of the active compounds

phosphate medium (see Table I) was better than ascitic fluid as a suspending medium for the cells The omission of $Ca^{++}$ from the medium permitted constant agitation of the cells during the incubation without clumping The same reaction in preparations of avian liver required the presence of $Mg^{++}$ or $Mn^{++}$ whereas $Ca^{++}$ was found to be inhibitory (85)

A relationship between the dose of amethopterin administered to leukemic mice and the capacity of the cell suspensions to form folinic acid *in vitro* was observed (Table I) The administration of a small dose (0 01 mg kg ) of amethopterin inhibited the reaction by approximately 50% It may be noted however that the dose (3 0 mg kg ) which Law (72 75) found necessary for the production of a significant antileukemic action is sufficient to produce complete inhibition of this reaction in cells harvested 3 hours after treatment (Table I) and may approximate the dosage necessary to maintain this inhibition throughout the interval (48 hours) between treatments

TABLE I

THE FORMATION OF FOLINIC ACID *in Vitro* BY ASCITIC LEUKEMIC CELLS (L 4946) FROM AMETHOPTERIN TREATED MICE

| Dose of amethopterin mg kg body weight | Folinic acid formed on incubation *in vitro* † γ/g cells dry weight | Per cent inhibition |
|---|---|---|
| – | 19 0 | 0 |
| 0 01 | 8 61 | 55 |
| 0 03 | 6 68 | 65 |
| 0 1 | 3 42 | 82 |
| 0 3 | 0 45 | 98 |
| 1 0 | 0 15 | 99 |

On the sixth day after the inoculation of AKR mice with an ascitic leukemia (L-4946) groups of mice were injected intraperitoneally with the different doses of amethopterin Three hours later the ascitic cells from each group (3 mice) were pooled and washed once in a Krebs Ringer phosphate buffer

† The leukemic cells (about 25 mg dry weight) were suspended in 2 0 ml of a Krebs Ringer phosphate medium pH 7 0 (modified by the omission of calcium chloride and by increasing the strength of the buffer to 0 05 M) containing glucose (0 01 M) DL-serine (0 01 M) sodium ascorbate (0 01 M) and pteroylglutamic acid (0 2 μmoles/ml ) The incubation was carried out with constant agitation under an atmosphere of nitrogen at 37 for 2 hours The vessels were then heated immediately at 100 for 30 minutes and the samples were prepared for microbial assay of the folinic acid activity (83) The results were corrected for the content of the inactive isomer present in the synthetic reference standard An amethopterin resistant strain of *Pediococcus cerevisiae* 8081 was used when an effect of the antagonist on the usual assay organism might be anticipated

The relationship between the concentration of amethopterin present in the suspending medium and the amount of folinic acid formed during the incubation of the suspensions of ascitic cells (L 1210 azaguanine dependent) is shown in Fig 3 This system *in vitro* is highly sensitive (50% inhibition by 0 05 γ/ml ) to amounts of amethopterin which can be expected to occur in the plasma and body fluids as a result of treatment with the drug (19 40) This technique is also applicable to the

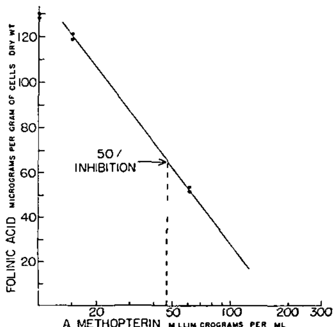



FIG 3 The sensitivity to amethopterin of the formation of folinic acid by ascitic leukemic cells incubated with pteroylglutamic acid

measurement of the sensitivity to amethopterin of this reaction in peripheral cells of leukemic patients (Table II)[3] Considerable information will be required before such sensitivity can be correlated with the effectiveness of treatment with amethopterin

## Comparison in Vitro of Closely Related Lines of Leukemia

A collaborative study with Dr Lloyd W Law of the National Cancer Institute was undertaken to determine whether the differences in sensitivity to treatment with amethopterin *in vivo* of certain sensitive and

[3] The generous assistance of Dr David H Clement and the staff of the Pediatrics Service of the Grace New Haven Community Hospital is gratefully acknowledged

resistant lines of leukemia in mice could be correlated with studies *in vitro* using these techniques

The 8 azaguanine resistant (L 1210 8AG R) and dependent (L 1210 AG D) sublines of leukemia were much more sensitive to treatment with amethopterin as indicated by survival time than was the parent line (L-1210-S) (74 77) Washed ascitic cells of the 8 azaguanine dependent line and the sensitive line were incubated with synthetic folic acid using the system just described The 8 azaguanine dependent line formed

TABLE II

THE SENSITIVITY TO AMETHOPTERIN OF THE FORMATION OF FOLINIC ACID BY HUMAN LEUKEMIC CELLS INCUBATED WITH FOLIC ACID

| Added to suspending medium | | Folinic acid formed during incubation γ/10[10] cells | |
|---|---|---|---|
| Folic acid 0 2 μM /ml | Amethopterin γ ml | Patient R H | Patient C M |
| — | — | 0 08 | 0 13 |
| + | — | 2 47 | 5 20 |
| + | — | 2 75 | — |
| + | 0 05 | — | 2 60 (51) |
| + | 0 1 | 0 68 (76) | 1 14 (80) |
| + | 0 3 | 0 19 (96) | 0 55 (93) |

The buffy layer from a sample of heparinized blood (20 ml) was washed once in Krebs-Ringer phosphate buffer Because of the limited amount of material cell counts of the suspensions used for each incubation were made directly Patient R H was an 8-year old boy with acute leukemia (white count 225 000) Patient C M was a 4 year-old boy with acute leukemia (white count 120 000) The conditions for the incubation were the same as those described for the preceding experiments The per cent inhibition of the reaction is indicated by the figures in parentheses

three to six times as much folinic acid as did the parent line (Table III) Because of a variable number of erythrocytes in preparations of the sensitive line the calculations were based on cell counts of the suspensions as well as on determinations of the dry weight of the cells The addition of erythrocytes to other suspensions (L 4946) in comparable experiments did not alter the yield of folinic acid which was observed. The results based on cell counts agreed substantially with the activity based on the dry weight of the cells (Table III)

The sensitivity of this system to amethopterin (Fig 3) was studied also in these two lines of leukemia and in lines resistant and sensitive to azaserine (Table IV) The preparation of the cells the incubations and the microbial assays for each comparison were carried out simultaneously In each case the concentration of amethopterin which in

hibited the formation of folinic acid by 50% was determined from a plot (see Fig 3) based on five to eight points The slope of the line was the same in each comparison only the position of the line was changed The 8 azaguanine dependent line was inhibited by less than half the concentration of amethopterin that inhibited the parent line by 50% The difference is more marked if the comparison is related to the amount of

TABLE III

COMPARISON OF THE ABILITY OF TWO CLOSELY RELATED LINES OF LEUKEMIC CELLS TO FORM FOLINIC ACID WHEN INCUBATED WITH FOLIC ACID

| | | Folinic acid formed † γ/g cells dry weight | | Activity azaguanine dependent / sensitive | |
|---|---|---|---|---|---|
| | | | L 1210 | | |
| Experiment | Folic acid added | L 1210 Sensitive | Azaguanine dependent | Dry weight | Cell count |
| 1 | — | 0 52 | 2 87 | | |
| | + | 23.5 | 146 | 6 2 | — |
| 2 | — | 0 65 | 0 95 | | |
| | + | 46 5 | 153 | 3 3 | 2 9 |
| 3 | — | 1 33 | 2 10 | | |
| | + | 23 7 | 130 | 5 5 | 3 9 |

In experiments 1 and 3 the ascitic cells were obtained from $CDF_1$ mice in experiment 2 from DBA/2 mice

† The medium and the conditions for the incubation were the same as those described in Table I

folinic acid represented by a 50% yield (see Table III) The azaserine *resistant line required only a slightly higher concentration* of amethopterin than the parent line to achieve a comparable degree of inhibition Although the significance of these small differences has not been evaluated the trend is as would be expected if the azaserine resistant cells had greater capacity to utilize exogenous purine derivatives which are products of the pathways *de novo* known to be sensitive to both azaserine and amethopterin It is of interest that 50% inhibition of the reaction in both the azaserine sensitive and resistant lines occurred at higher concentrations than in the L 1210 leukemias The sensitivity of these lines to amethopterin *has not been evaluated*

These preliminary findings indicate that differences between closely related lines of leukemia (L 1210 S and L 1210 8AG D) with respect to the metabolism of folic acid are measurable *in vitro* by these techniques

These studies are being extended to other leukemias particularly those sensitive and resistant to amethopterin

One may speculate that the increased capacity of the 8 azaguanine dependent cells to form folinic acid is associated with a greater utiliza tion of metabolic reactions requiring the functional forms of folic acid The greater sensitivity of these cells to amethopterin *in vivo* is strong evidence that folic acid is more essential for the growth and multiplica

TABLE IV
COMPARISON OF THE SENSITIVITY TO AMETHOPTERIN OF THE FORMATION OF FOLINIC ACID FROM FOLIC ACID BY CLOSELY RELATED LINES OF LEUKEMIC CELLS

| Ascitic cells | Concentration of amethopterin required for 50% inhibition of folinic acid formation mμ ml of suspending fluid | |
|---|---|---|
| | Experiment 1 | Experiment 2 |
| L-1210 | | |
| Azaguanine sensitive | 110 | 75 |
| Azaguanine dependent | 45 | 32 |
| Plasma cell 70429 | | |
| Azaserine sensitive | 128 | 12_ |
| Azaserine resistant | 176 | 174 |

The medium and the conditions for the incubation were the same as described in Table I and Fig 3

tion of these cells (L-1210-8AG D) than for those of the parent line The sensitivity of the 8-azaguanine resistant and dependent cells to ameth opterin appears to be more than a casual characteristic associated with the selection of these cells The same altered sensitivity of leukemic cells to amethopterin was associated with resistance to other purine antagonists (77 96) and similar collateral sensitivity was observed in bacterial studies (35 50 54 93)

## Changes in Metabolic Patterns Associated with Resistance

*Streptococcus faecalis* can grow well in a medium lacking purines provided that folic acid is supplied Purines are used readily by this organism however if present in the medium (54 55) Similarly mam malian cells both normal and neoplastic have the capacity to synthesize *de novo* the purine components of nucleic acids as measured by the incorporation of $C^{14}$ labeled formate or glycine and also can utilize

purines as indicated by the incorporation of $C^{14}$ labeled adenine or guanine into the nucleic acids of the cells (5 37 98) Considerable evidence suggests that the ability to alter the proportion of the total nucleic acids derived from the *de novo* pathways of synthesis or from pathways involving the utilization of preformed purines of exogenous origin is of importance in the development of resistance to antagonists which can interfere with these metabolic pathways If a resistant cell circumvents the effect of analogs of components of nucleic acids by alterations resulting in a decreased uptake of these agents such changes may also sacrifice the capacity to take up the corresponding metabolites thereby requiring that the need for such metabolites be supplied by an increase in the synthesis *de novo* of such compounds if the rate of growth of the cell is to be maintained The role of folic acid in those reactions involving the transfer of formate in the biosynthesis of purine derivatives *de novo* has been well defined (13 46-49)

Many studies since the early experiments of Ehrlich (31) have shown that a decreased uptake of different drugs can be associated with resistance in microorganisms The careful studies bv Eagle (29 30) of the binding of penicillin by bacteria require reservation in attributing such changes to impermeability rather than a decreased binding capacity of the cytoplasm One striking observation concerning tubercle bacilli which were resistant to isoniazid indicated that the resistant organisms unlike the sensitive strains did not take up isoniazid when grown in medium containing the $C^{14}$ labeled drug (4) More specifically in relation to nucleic acid antagonists Elion and co workers (33-35) observed in strains of *L casei* that resistance to diaminopurine was associated with a decreased capacity to take up adenine and diaminopurine also a 6 mercaptopurine resistant strain was virtually unable to use hypoxanthine for growth and had a higher requirement for folic acid Handschumacher and Welch (52) found that a 6 azauracil resistant strain of *S faecalis* in contrast to the parent strain had very limited ability to take up uracil 2 $C^{14}$ from the medium

In several instances resistant organisms were found to have a requirement for products of the inhibited reactions Presumably interference by amethopterin with the synthesis of purine components of nucleic acids *de novo* resulted in the selection of amethopterin resistant strains which required preformed purines for growth even in the presence of folic acid (54) It is of interest that the requirement of this resistant strain (*S faecalis*/A) for folic acid was about one third that of the parent strain (20 90) Also a strain of *L casei* which was selected for resistance to amethopterin while growing in crude medium was found

to have developed a requirement for thymine (2)

The hypothesis that a shift in the pattern of metabolism between synthesis *de novo* and utilization of metabolites of exogenous origin which are products of the inhibited reactions may be of major importance in the development of resistance is worthy of detailed examination When drugs such as amethopterin or azaserine inactivate certain enzymes involved in the biosynthesis *de novo* of purine derivatives the cell may circumvent the blocked reactions by the development of an alternate pathway leading to the formation of the same product from simple precursors within the cell or by the utilization of such products which may

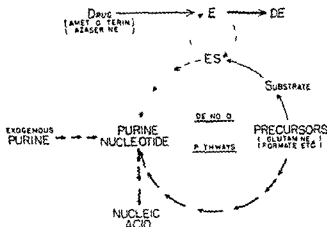



Fig 4 The effect of a drug on the balance between the pathways of biosynthesis *de novo* and the utilization of exogenous metabolites

be present in the surrounding medium or body fluids (Fig 4) The ability to develop an alternate pathway *de novo* appears to be limited On the other hand interference with the utilization of exogenous purine derivatives may result in greater dependency on reactions *de novo* for growth and survival The 8 azaguanine dependent cells were only twice as sensitive to amethopterin *in vitro* as were the cells of the parent line as measured by this technique yet amethopterin increased markedly the survival time of mice carrying the 8 azaguanine dependent line (74 77) These experiments suggest that the actual difference in the concentration of a drug affecting the sensitive and resistant populations of leukemic cells (see Fig 1) may be small in contrast to the thousandfold differences common in bacterial studies Therefore it is suggested that slight changes in metabolism may be capable of conferring resistance

upon the leukemic cells or conversely slight changes may be associated with a much more favorable selective action of a drug

### Other Possible Mechanisms of Resistance to Amethopterin

Although it is not possible to present in detail other studies pertinent to amethopterin resistance brief mention should be made of certain other mechanisms of resistance which could be involved

Jacobson (61 62) presented evidence that leukemic cells of mice inactivated aminopterin as measured by the mitotic arrest of tissue cultures Although there is good evidence that aminopterin can be inactivated by bacteria (103 104) Kieler and Kieler (65) were unable to detect any destruction of amethopterin by leukemic cells in tissue culture using a specific microbial assay Also Futterman (42) found that aminopterin inhibited the inactivation of pteroylglutamic acid by liver preparations Since inactivation of very small amounts of amethopterin could be associated with relatively large changes in the effective concentration the ability of leukemic cells to inactivate amethopterin requires more detailed investigation

The possibility that aminopterin can be bound in an inactive form was suggested by the finding that an amethopterin resistant strain of *S faecalis* actually took up much more aminopterin than the sensitive strain when resting cell suspensions were incubated with the drug in an appropriate medium (2, 3) Furthermore the growth of this resistant organism and its capacity to form folinic acid were inhibited more effectively by 4 amino 10-methylpteroic acid than by amethopterin even though complete cross resistance between these two closely related compounds would have been expected (2 3)

The involvement of an increased ability to form a metabolite which can compete more effectively with the antagonist was implied by the finding that the capacity of amethopterin resistant strains of *S faecalis* to form folinic acid (10 90) was related directly to the degree of resistance to amethopterin (2 3)

Certain species of animals particularly the rabbit and the guinea pig are remarkably refractory to the toxic effects of the folic acid antagonists (44 79) An attempt to explain this "natural resistance" indicated little difference in the rate of excretion of aminopterin by rats and guinea pigs (107) However the ability of preparations of rat liver to form folinic acid was lost rapidly on dialysis whereas similar preparations of guinea pig liver treated in the same way retained this activity (107) Although the factors concerned with the stability of the preparations require detailed study it is a reasonable possibility that a greater relative

affinity of the enzyme of one species for the derivatives of folic acid could contribute to the ability of the guinea pig to tolerate fifty times the dose of aminopterin parenterally which is lethal for the rat

## Conclusion

The occurrence of drug resistance is of greater significance with regard to antileukemic agents than with reference to antibiotics because of the ineffectiveness of the defense mechanisms of the body against neoplastic cells and because of the limited selective action of those agents which are available at present The occurrence of cross resistance may limit considerably the number of new useful compounds Many of the possible mechanisms by which leukemic and other cells circumvent the initial effectiveness of certain drugs can be approached experimentally The elucidation of the biochemical basis of drug resistance is a challenging problem deserving of more intensive investigation Such studies can afford a unique opportunity to gauge the flexibility of the metabolic patterns of mammalian cells which are related to the basis for selective toxicity More detailed knowledge of the metabolic reactions associated with changes in sensitivity to antileukemic drugs may suggest means by which the limited usefulness of present agents may be increased.

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### REFERENCES


1 Albrecht A M Fede ation P oc 15 210 (1958)
2 Anton A H Doctorate Dissertation Yale University 1956
3 Anton A H and Nichol C A Proc Am Assoc Cancer Research 2 91 (1956)
4 Barclay W R Ebert R H and Koch Weser D Am Rev Tuberc 67 490 (1952)
5 Bennett L L Jr Skipper H E Stock C C and Rhoads C P Cancer Research 15 485 (1955)
6 Bethell F H Harris R S and Stokstad E L R in "The Vitamins" (W H Sebrell and R S Harris eds ) Vol 3 p 87 Academic Press New York 1954
7 Blakley R L Nature 173 729 (1954)
8 Blakley R L Biochem J 58 448 (1954)
9 Briggs G M and Daft F S Ann Rev Biochem 24 339 (1955)
10 Broquist, H P Kohler A R Hutchison D J and Burchenal J H J Biol Chem 202 59 (1953)
11 Bryson V and Demerec M Ann N Y Acad Sci 53 283 (1950)

12 Bryson V and Szybalski W *Advances in Genetics* 7 1 (1955)
13 Buchanan J M this volume p 523
14 Burchenal J H *J Clin Invest* 29 801 (1950)
15 Burchenal J H *Cancer Research* 14 615 (1954)
16 Burchenal J H *in* Leukaemia Research (G L W Wolstenholme and M I Cameron eds ) p 13 Ciba Foundation Symposium J & A Churchill Ltd London 1954
17 Burchenal J H *Am J Clin Nutrition* 3 311 (1955)
18 Burchenal J H Robinson E Johnston S F and Kishida M N *Science* 111 116 (1950)
19 Burchenal J H Waring G B Ellison R R and Reilly H C *Proc Soc Exptl Biol Med* 78 603 (1951)
20 Burchenal J H Waring G B and Hutchison D J *Proc Soc Exptl Biol Med* 78 311 (1951)
21 Burchenal J H Webber L F Meigs G M and Biedler J L *Blood* 6 337 (1951)
22 Cavalli Sforza L L and Lederberg J *6th Intern Congr Microbiol Rept Proc* 108 (1953)
23 Clarke D A Philips F S Sternberg S S Stock C C Elion G B and Hitchings G H *Cancer Research* 13 593 (1953)
24 Danielli J I *in* Leukaemia Research (G E W Wolstenholme and M P Cameron eds ) p 263 Ciba Foundation Symposium J & A Churchill Ltd London 1954
25 Davis B D and Maas W K *Proc Natl Acad Sci* (*U S*) 38 775 (1952)
26 Dean A C R *in* Origin of Resistance to Toxic Agents" (M G Sevag R D Reid and O E Reynolds eds ) p 42 Academic Press New York 1955
27 Demerec M *J Bacteriol* 56 63 (1948)
28 Eagle H *Ann N Y Acad Sci* 59 243 (1954)
29 Eagle H *J Exptl Med* 99 207 (1954)
30 Eagle H *J Exptl Med* 100 103 (1954)
31 Ehrlich P *Lancet* ii 445 (1913)
32 Elion G B and Hitchings G H *Proc Am Assoc Cancer* (1953)
33 Elion G B Singer S and Hitchings G H *J Biol Che*
34 Elion G B Singer S Hitchings G H Balis M F *J Biol Chem* 202 647 (1953)
35 Elion G B Singer S and Hitchings G H *Ann* (1954)
36 *Farber S Blood* 7 97 (1952)
37 Fernandes J F LePage G A and Lindner (1956)
38 Finland M *New Engl J Med* 252 570 (
39 Finland M *New Engl J Med* 253 909 9
40 Fountain J R Waring G B Hutchison *Soc Exptl Biol Med* 81 193 (1952)
41 Furth J and Kahn M C *Am J Cancer*
42 Futterman S *Federation Proc* 16 258 (
43 George M and Pandalai K M *Lancet*

44 Girdwood R H *Brit J Nutrition* **5** 1 (1951)
45 Goldin, A Mantel N Greenhouse S W Venditti J M and Humphreys S R *Cancer Research* **14** 43 (1954)
46 Goldthwait D A this volume p 505
47 Greenberg, G R *Federation Proc* **13** 745 (1954)
48 Greenberg, G R *J Am. Chem Soc* **76** 1458 (1954)
49 Greenberg, G R Jaenicke L. and Silverman M *Biochim et Biophys Acta* **17** 589 (1955)
50 Guthrie R Holland J F Hyatt E A Hillman M and Mount D T *Proc Am Assoc Cancer Research* **2** 113 (1956)
51 Handschumacher R E and Welch A D *Federation Proc* **15** 267 (1956)
52 Handschumacher R E and Welch A D personal communication
53 Hess A D *Am J Trop Med Hyg* **1** 371 (1952) **2** 311 (1953)
54 Hutchison D J *Ann N Y Acad Sci* **60** 212 (1954)
55 Hutchison D J this volume p 605
56 Hutchison D J and Burchenal J H *Proc Soc Exptl Biol Med* **80** 516 (1952)
57 Hutchison D J and Burchenal J H *in* Chemistry and Biology of Pteridines (G E W Wolstenholme and M P Cameron, eds ) p 368 Ciba *Foundation Symposium Little Brown and Company Boston*, 1954
58 Hotchkiss R. D *Cold Spring Harbor Symposia Quant Biol* **16** 457 (1951)
59 Hotchkiss R D *in* Phosphorous Metabolism" (W D McElroy and B Glass eds ) Vol II p 426 Johns Hopkins Press Baltimore 1952
60 Hotchkiss R D and Marmur J *Proc Natl Acad Sci U S* **40** 55 (1954)
61 Jacobson W *J Physiol* **123** 618 (1954)
62 Jacobson W *in* Chemistry and Biology of Pteridines (G E W Wolstenholme and M P Cameron eds ) p 329 Ciba Foundation Symposium Little Brown and Company Boston 1954
63 Jaenicke L. *Biochim et Biophys Acta* **17** 588 (1955)
64 Jukes T H and Williams W L *in "The Vitamins"* (W H Sebrell and R S Harris eds ) Vol I p 448 Academic Press New York 1954
65 Kieler J and Kieler E *Cancer Research* **14** 428 (1954)
66 Kisliuk R L and Sakami W *J Am Chem Soc* **76** 1456 (1954)
67 Law L W *Proc Soc Exptl Biol Med* **77** 340 (1951)
68 Law L W *Proc Soc Exptl Biol Med* **78** 499 (1951)
69 Law L W *J Natl Cancer Inst* **11** 849 (1951)
70 Law L W *Cancer Research* **12** 871 (1952)
71 Law L W *Nature* **169** 628 (1952)
72 Law L W *Texas Repts Biol and Med* **10** 571 (1952)
73 Law L W *Cancer Research* **14** 695 (1954)
74 Law L W *in* Leukaemia Research (G E W Wolstenholme and M P Cameron eds ) p 105 Ciba Foundation Symposium J & A Churchill, Ltd London 1954
75 Law L W personal communication
76 Law L W and Boyle P J *Proc Soc Exptl Biol Med* **74** 599 (1950)
77 Law L W Taormina, V and Boyle P J *Ann N Y Acad Sci* **60** 244 (1954)
78 Miller C P and Bohnhoff M *J Bacteriol* **54** 467 (1947)

12 Bryson V and Szybalski W *Advances in Genetics* **7** 1 (1955)
13 Buchanan J M this volume p 523
14 Burchenal J H *J Clin Invest* **29** 801 (1950)
15 Burchenal J H *Cancer Research* **14** 615 (1954)
16 Burchenal J H *in* Leukaemia Research (G E W Wolstenholme and M P Cameron eds) p 13 Ciba Foundation Symposium J & A Churchill Ltd London 1954
17 Burchenal J H *Am J Clin Nutrition* **3** 311 (1955)
18 Burchenal J H Robinson E Johnston S F and Kushida M N *Science* **111** 116 (1950)
19 Burchenal J H Waring G B Ellison R R and Reilly H C *Proc Soc Exptl Biol Med* **78** 603 (1951)
20 Burchenal J H Waring G B and Hutchison D J *Proc Soc Exptl Biol Med* **78** 311 (1951)
21 Burchenal J H Webber L F Meigs G M and Biedler J L *Blood* **6** 337 (1951)
22 Cavalli Sforza L L and Lederberg J *6th Intern Congr Microbiol Rept Proc* 108 (1953)
23 Clarke D A Philips F S Sternberg S S Stock C C Elion G B and Hitchings G H *Cancer Research* **13** 593 (1953)
24 Danielli J F *in* Leukaemia Research (G E W Wolstenholme and M P Cameron eds) p 263 Ciba Foundation Symposium J & A Churchill Ltd London 1954
25 Davis B D and Maas W K *Proc Natl Acad Sci (U S)* **38** 775 (1952)
26 Dean A C R *in* Origin of Resistance to Toxic Agents (M G Sevag R D Reid and O E Reynolds eds) p 42 Academic Press New York 1955
27 Demerec M *J Bacteriol* **56** 63 (1948)
28 Eagle H *Ann N Y Acad Sci* **59** 243 (1954)
29 Eagle H *J Exptl Med* **99** 207 (1954)
30 Eagle H *J Exptl Med* **100** 103 (1954)
31 Ehrlich P *Lancet* ii 445 (1913)
32 Elion, G B and Hitchings G H *Proc Am Assoc Cancer Research* **1** 13 (1953)
33 Elion G B Singer S and Hitchings G H *J Biol Chem* **204** 35 (1953)
34 Elion G B Singer S Hitchings G H Balis M E and Brown G B *J Biol Chem* **202** 647 (1953)
35 Elion G B Singer S and Hitchings G H *Ann N Y Acad Sci* **60** 200 (1954)
36 Farber S *Blood* **7** 97 (1952)
37 Fernandes J F LePage G A and Lindner A *Cancer Research* **16** 154 (1956)
38 Finland M *New Engl J Med* **252** 570 (1955)
39 Finland M *New Engl J Med* **253** 909 969 1019 (1955)
40 Fountain J R Waring G B Hutchison D J and Burchenal J H *Proc Soc Exptl Biol Med* **81** 193 (1952)
41 Furth J and Kahn M C *Am J Cancer* **31** 276 (1937)
42 Futterman S *Federation Proc* **15** 258 (1956)
43 George M and Pandalai K M *Lancet* **1** 955 (1949)

# 38

## Metabolic and Nutritional Variations in Drug Resistant *Streptococcus faecalis*[1]

DORRIS J HUTCHISON

Sloan-Kettering Institute and the Sloan-Kettering Division of Cornell University Medical College New York New York

The folic acid analog 4 amino 10-methylpteroylglutamic acid (amethopterin) and the adenine analog 6-mercaptopurine (6-MP) have been used somewhat successfully in the treatment of several types of leukemia, both in children and in adults (8 16) It has been presumed that information regarding some of the mechanisms by which these drugs act could be obtained through the study of their effects on the growth of *Streptococcus faecalis* ATCC 8043 Under certain conditions *S faecalis* requires a purine derivative for growth and under other conditions it requires folic acid or a related compound for growth (31) Furthermore since the development of drug resistant leukemic cells probably is the major factor which prevents the complete eradication of the disease by chemotherapy it was presumed that information regarding the alterations in drug resistant bacterial mutants might suggest mechanisms that could be operative in the drug resistant leukemic process

Drug resistance has been observed in innumerable bacterial systems and many mechanisms have been postulated for the explanation of the phenomenon (29) Davis and Maas (10) suggested seven mechanisms to explain the resistance to sulfonamides in *Escherichia coli* All the postulated mechanisms except the concept of increased affinity of enzyme for metabolite versus antimetabolite were shown not to be applicable to this mutant Eagle (11) demonstrated in several bacteria that mechanisms other than detoxification are responsible for penicillin

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79 Minnich V Moore C V Smith D E and Elliott G V *Arch Pathol* 50 787 (1950)
80 Mitchison D A *Brit Med Bull* 10 115 (1954)
81 Murphy M L Ellison R R Karnofsky D A and Burchenal J H *J Clin Invest* 33 1388 (1954)
82 Newcombe H B *in* Origins of Resistance to Toxic Agents (M G Sevag R D Reid and O E Reynolds eds ) p 4 Academic Press New York 1955
83 Nichol C A *Cancer Research* 14 522 (1954)
84 Nichol C A *in* Symposium on Vitamin Metabolism National Vitamin Foundation New York 1956
85 Nichol C A unpublished data
86 Nichol C A Anton A H and Zakrzewski S F *Science* 121 275 (1955)
87 Nichol C A and Law L W unpublished data
88 Nichol C A and Welch A D *Proc Soc Exptl Biol Med* 74 403 (1950)
89 Nichol C A and Welch A D *in* Symposium on Antimetabolites and Cancer (C P Rhoads ed ) p 63 American Association for the Advancement of Science Washington D C 1955
90 Nichol C A Zakrzewski S F and Welch A D *Proc Soc Exptl Biol Med* 83 272 (1953)
91 Novick A and Szilard L *Nature* 170 926 (1952)
92 Potter M *Proc Am Assoc Cancer Research* 2 140 (1956)
93 Schabel F M Jr Wheeler G P and Skipper H E *Proc Am Assoc Cancer Research* 2 43 (1955)
94 Sevag M G Reid R D and Reynolds O R eds Origins of Resistance to Toxic Agents" Academic Press New York 1955
95 Silverman M Ebaugh F G and Gardiner R C *Federation Proc* 15 356 (1956)
96 Skipper H E *Ann N Y Acad Sci* 60 267 (1954)
97 Skipper H E *in* Symposium on Antimetabolites and Cancer" (C P Rhoads ed ) p 219 American Association for the Advancement of Science Washington D C 1955
98 Skipper H E Bennett L L Jr and Law L W *Cancer Research* 12 677 (1952)
99 Szybalski W *Antibiotics & Chemotherapy* 3 1095 (1953)
100 Syzbalski W and Bryson V *J Bacteriol* 64 489 (1952)
101 Syzbalski W and Bryson V *J Bacteriol* 66 468 (1953)
102 Vourcka A *Lancet* i 62 (1948)
103 Webb M *in* Chemistry and Biology of Pteridines (G E W Wolstenholme and M I Cameron eds ) p 253 Ciba Foundation Symposium Little Brown and Company Boston 1954
104 Webb M *Biochim et Biophys Acta* 17 258 (1956)
105 Welch, A D and Nichol C A *Ann Rev Biochem* 21 633 (1952)
106 Winner H I *Lancet* i 674 (1948)
107 Wood D R personal communication
108 Zakrzewski S F and Nichol C A *J Biol Chem* 205 361 (1953)
109 Zakrzewski S F and Nichol C A *Federation Proc* 14 311 (1955)
110 Zakrzewski S F and Nichol C A *Federation Proc* 15 390 (1956)

was noted between mutants SF MP and SF/MP A and the parent culture with the exception that thymine supplementation did not spare the amount of PGA requirement for each mutant. SF/A was quantitatively different from the other organisms five times as much PGA was required in the F PP medium and supplementation with thymine did not spare the PGA during 42 hours but did in 70 hours whereas the inclusion of the purines and uracil spared the PGA requirement by fifty fold and this was attained in 18 hours

The amount of CF (expressed in terms of natural CF) required by each of the strains (Table II) under the same growth conditions as

TABLE II

QUANTITATIVE GROWTH REQUIREMENTS FOR CITROVORUM FACTOR IN THREE MEDIA

| | Amount of CF required for HMG mγ ml | | |
|---|---|---|---|
| *S faecalis* | F PP | F PP+T | F |
| O | 0.8 | 0.75 | 0.4 |
| A | 4.0 | 5.0† | 0.3 |
| MP | 0.75 | 0.75 | 0.2 |
| MP/A | 0.5 | 0.5 | 0.2 |

42 hours
† 70 hours

described for PGA showed that CF played a role similar to that of PGA in the growth of SF O an observation first reported by Broquist *et al* (4) and subsequently by many investigators The response of SF/MP and SF MP/A to CF was essentially the same as to PGA except that a slightly lower amount of CF was required by SF/MP A in all media SF/A responded to CF as it did to PGA except in the purine medium where less PGA was required

Thymine or thymidine and a natural purine were first shown by Stokes (31) to replace completely the PCA requirement of SF/O The quantitative requirements (Table III) of each in the F medium indicate that thymidine was a better growth factor for SF/O and SF MP/A and about equimolar amounts were required by the other two mutants All the mutants required less of thymine or thymidine than the parent strain

The sparing effect of purines on the PGA requirement of these bacteria suggested that some differences might occur in their growth response to some free purine bases such as adenine guanine xanthine hypoxanthine 6 mercaptopurine 2,6 diaminopurine (2 6-DAP) and isoguanine The growth response on these purines in the F PP+PGA medium is shown in Table IV There was little or no growth stimulation

resistance Some resistant strains which do not produce penicillinase were found to bind more sulfur labeled penicillin than sensitive strains whereas in other cases there was less binding by resistant mutants and in others there was equal binding by the sensitive and resistant strains

In our studies on drug resistance in bacteria some observations have been made on a number of postulated mechanisms for resistance Data pertinent to the mechanisms of metabolite requirements alternate path ways of nucleic biosynthesis and enzyme activity will be considered The bacteria used for these experiments were *S faecalis* ATCC 8043 (SF/O) an amethopterin resistant mutant SF/A (9) a 6 MP resistant mutant SF/MP (22) and a mutant resistant to both 6-MP and amethop terin SF/MP/A This double mutant was isolated from SF/MP by serial transfer in the F PP (free of purines and pyrimidines) + PGA medium (17) supplemented with amethopterin

Presumably a mutant could become resistant to a drug such as amethopterin or 6 MP if it no longer required as much of a specific metabolite for example pteroylglutamic acid (PGA) or a natural purine as its sensitive parent strain The inhibitory effect of the antimetabolite would not be so profound in this situation

The quantitative growth requirements of four strains of *S faecalis* for PGA were determined after 18 hours the results are presented in Table I

TABLE I
QUANTITATIVE GROWTH REQUIREMENTS FOR PGA IN THREE MEDIA

| | Amount of PGA required for HMG mγ/ml | | |
|---|---|---|---|
| *S faecalis* | F PP | F PP + T | F |
| O | 1 0 | 0 5 | 0 3 |
| A | 5 0 | 1 0† | 0 1 |
| MP | 1 0 | 1 0 | 0 25 |
| MP/A | 1 0 | 1 0 | 0 3 |

42 hours
† 70 hours

The three media used were *(1)* F *(Flynn et al 17)* *(2)* F PP (F medi um without adenine guanine xanthine and uracil supplementation) and *(3)* F PP + T (F PP medium supplemented with 1 γ/ml thymine The amount of PGA required for half maximum growth (HMG) of SF/O was 1 mγ/ml in the F PP medium thymine supplementation of this medium decreased the PGA requirement to one half A threefold sparing effect on PGA was observed when the medium was supplemented with purines and uracil A similarity in quantitative PGA requirement

with these strains of S faecalis show a number of quantitative differences in each organism in regard to its utilization of PGA CF thymine and purines and furthermore that different alterations occur in mutants resistant to the same drug Conversely different changes occur in mutants resistant to the same drugs—for example the stimulatory effect of adenine on SF/A and the inhibitory effect of the same concentration of adenine on SF/MP/A or the inability of SF/MP to grow on adenine guanine and hypoxanthine and the converse in the case of SF/MP/A A decrease in the amount of thymine required occurred in all three mutants the PGA response was mostly altered in SF/A and the CF response was altered most in SF/MP/A

TABLE V

Growth Response to Purines in F PP+Thymine Medium

| Supplements 0.3 mM | SF/O | SF/A | SF/MP | SF/MP/A |
|---|---|---|---|---|
| | OD | | | |
| None | 0 | 0 | 0 | 0 |
| Adenine | 0 37 | 0 40 | 0 | 0 |
| Guanine | 0 30 | 0 34 | 0 | 0 34 |
| Hypoxanthine | 0 38 | 0 39 | 0 | 0 22 |
| Xanthine | 0 43 | 0 45 | 0 54 | 0 32 |
| Isoguanine | 0 15 | 0 12 | 0 13 | 0 11 |
| 2 6-DAP | 0 | 0 | 0 | 0 10 |
| 6-MP | 0 15 | 0 17 | 0 03 | 0 10 |

Growth at lower levels of adenine

PGA requirements in purine pyrimidine free (F PP) thymine supplemented (F PP+T) and purine pyrimidine supplemented (F) media suggested a marked dichotomy of the *de novo* and preformed pathways of nucleic acid biosynthesis The alterations in these mutants served as a basis for collaborative studies with Dr M Earl Balis concerned with the utilization of labeled exogenous purines and the interconversion of these purines into purines of pentosenucleic acid (PNA) The growth phase of these "incorporation experiments was such that the organism could use a purine per se or synthesize purine derivatives from small molecules through folic acid mediated reactions The methods used are similar to those which have been described by Balis *et al* (1) for *Escherichia coli* SF/O used exogenous adenine and guanine for both PNA adenine and guanine adenine was a slightly better precursor (Table VI) The relative specific activity of the PNA adenine and guanine indicate that about equal synthesis was carried out by the *de*

of SF/O SF/MP or SF/MP/A by any of the purines There was some inhibition of SF/O by 6 MP and 2 6 DAP SF/A which did not grow in the F PP+PGA medium in 24 hours grew on all the purines except 2 6 DAP The growth on 6 MP was about one half that obtained on the other purines

TABLE III
QUANTITATIVE GROWTH REQUIREMENTS FOR THYMINE AND THYMIDINE (F MEDIUM)

| S faecalis | Amounts required for HMG γ/ml | |
|---|---|---|
| | Thymine | Thymidine |
| O | 0 43 | 0 52 |
| A | 0 20 | 0 48 |
| MP | 0 15 | 0 40 |
| MP/A | 0 30 | 0 40 |

TABLE IV
GROWTH RESPONSES TO PURINES IN F PP+PGA MEDIUM

| Supplements 0 03 mM | SF/O | SF/A | SF/MP | SF/MP/A |
|---|---|---|---|---|
| | O D | | | |
| None | 0 40 | 0 | 0 39 | 0 31 |
| Adenine | 0 47 | 0 40 | 0 41 | 0 37 |
| Guanine | 0 44 | 0 33 | 0 41 | 0 34 |
| Hypoxanthine | 0 42 | 0 33 | 0 38 | 0 40 |
| Xanthine | 0 47 | 0 30 | 0 49 | 0 41 |
| Isoguanine | 0 42 | 0 30 | 0 40 | 0 35 |
| 2 6-DAP | 0 | 0 | 0 40 | 0 39 |
| 6-MP | 0 30 | 0 18 | 0 40 | 0 34 |

The response of these bacteria to purine bases is more clearly delineated in the F PP + thymine medium since in this medium the baseline of a lack of growth in the absence of a purine is the same for all cultures (Table V) Adenine guanine hypoxanthine and xanthine were good purine sources for SF/O but 6 MP gave a slight growth response (the presence of traces of hypoxanthine could account for this growth) and isoguanine was also a poor purine source SF/A was similar to the parent strain SF/MP grew well on xanthine and slightly on isoguanine the lack of growth on 6-MP is noteworthy SF/MP/A was inhibited by the concentration of adenine used in these experiments at one third the concentration the growth was comparable to that obtained on guanine SF/MP/A grew with exogenous 6 MP or 2 6 DAP

The myriad changes which have been observed in the growth studies

DAP as a purine source it is resistant to it Despite these increases in resistance to purine analogs SF/MP/A was more sensitive to unsubstituted purine than any of the other organisms including the parent culture The increased relative effectiveness of amethopterin on SF/A in the F PP+PGA medium further indicates the mutant's weakness in carrying out *de novo* synthesis while the other two mutants SF/MP and SF/MP/A were not markedly affected by the absence of natural purines this is in agreement with the postulate that they have a slight preference for *de novo* synthesis

TABLE VII
GROWTH INHIBITION AND CROSS RESISTANCE

| | F+PGA medium | F PP+PGA medium | | | |
|---|---|---|---|---|---|
| | amethopterin | Amethopterin | 6-MP | 2 6-DAP | Purine |
| *S faecalis* | γ/ml. for 50% inhibition | | | | |
| O | 0 001 | 0 0005 | 10 | 3 | 20 |
| A | 300 | 60 | 1 | 25 | 20 |
| MP | 0 004 | 0 003 | >500 | 30 | _0 |
| MP/A | 300 | _00 | >500 | _00 | 10 |

The enzymatic formation of an increased amount of a specific metabolite such as CF capable of competing with amethopterin might be a process responsible for resistance in antifolics The conversion of folic acid to CF *in vivo* as demonstrated by Bleiler *et al* (2) Sauberlich (28) Broquist *et al* (6) Girdwood (19) and others and *in vitro* by Nichol (25) Nichol and Welch (26) Broquist *et al* (5) Hendlin *et al* (21) Foley and Haley (18) and others suggested that CF might be the biologically active form of folic acid Recent studies by Greenberg (20) Kisliuk and Sakami (24) Silverman and Keresztesy (30) Nichol *et al* (27) and Bolinder *et al* (3) suggest that CF may not be the active formylating factor in nature CF may indeed be the heat stable form of the biologically active factor

Resting cells of SF/A were shown (5 23) to form more CF from PGA than did SF/O When glucose supplementation of the resting cell mixture was studied (Table VIII) washed cells of SF/O had the same capacity to form CF as did SF/A The enzymatic activity of SF/A cells was not appreciably affected by the presence of glucose

Studies on enzyme activity have been done with *S faecalis* strains relative to rates of CF formation The conditions used in these resting cell experiments were those that were optimal for SF/O (Table IX)

*novo* route in the presence of exogenous adenine or guanine SF/A *incorporated* more of each exogenously supplied purine into PNA adenine and guanine than did SF/O This is in agreement with the stimulatory *effect* of adenine and guanine observed in the F PP+PGA medium *growth studies* SF/A utilized exogenous purines to a greater extent than SF/O but the *de novo* route of nucleic acid synthesis is still very much in effect SF/MP utilized exogenous adenine slightly for PNA adenine and exogenous guanine only poorly for PNA guanine and the interconversion was negligible Since the growth of SF/MP in these experiments was comparable to that of the other two cultures it is

TABLE VI
BIOSYNTHESIS OF PENTOSENUCLEIC ACID ADENINE AND GUANINE

| | Exogenous adenine | | Exogenous guanine | |
|---|---|---|---|---|
| | Relative specific activity of PNA | | | |
| *S faecalis* | Adenine | Guanine | Adenine | Guanine |
| O | 48 | 44 | 39 | 41 |
| A | 58 | 65 | 55 | 56 |
| MP | 19 | 4 | 1 | 12 |
| MP/A | 36 | 51 | 39 | 45 |

• Relative specific activity = $\frac{\text{counts per minute per mole of isolated compound}}{\text{counts per minute per mole of added compound}} \times 100$

reasonable to suggest that SF/MP is unable to use adenine and guanine for the biosynthesis of PNA purines SF/MP/A was similar to the parent culture showing a slight preference for the *de novo* pathway

Increased resistance to a drug regardless of the mechanism accompanied by a concurrent increase in sensitivity to another drug would be an ideal system for the chemotherapist This phenomenon has been observed in antibiotic resistance in a number of bacterial studies (32) and in 6 MP and 2 6 DAP resistant *L casei* (13 14) The measurement of the comparative inhibitory effects of amethopterin 2 6 DAP 6 MP and unsubstituted purine on the strains of *S faecalis* (Table VII) show that in SF/A resistance to amethopterin resulted in an increased sensitivity to 6 MP an increased resistance to 2 6 DAP and no change for unsubstituted purine Resistance to 6-MP in SF/MP was accompanied by a slight increase in resistance to amethopterin and 2 6 DAP with no change in respect to unsubstituted purine SF/MP/A by its nature was resistant to 6-MP and amethopterin and since SF/MP/A can use 2 6

DAP as a purine source it is resistant to it. Despite these increases in resistance to purine analogs SF/MP/A was more sensitive to unsubstituted purine than any of the other organisms including the parent culture. The increased relative effectiveness of amethopterin on SF/A in the F PP+PGA medium further indicates the mutant's weakness in carrying out *de novo* synthesis while the other two mutants SF/MP and SF/MP/A were not markedly affected by the absence of natural purines; this is in agreement with the postulate that they have a slight preference for *de novo* synthesis.

TABLE VII

GROWTH INHIBITION AND CROSS RESISTANCE

| *S faecalis* | F+PGA medium amethopterin | F PP+PGA medium Amethopterin | 6-MP | 2 6-DAP | Purine |
|---|---|---|---|---|---|
| | | γ/ml for 50% inhibition | | | |
| O | 0 001 | 0 0005 | 10 | 3 | 20 |
| A | 300 | 60 | 1 | 25 | 20 |
| MP | 0 004 | 0 003 | >500 | 30 | 20 |
| MP/A | 500 | 200 | >500 | 200 | 10 |

The enzymatic formation of an increased amount of a specific metabolite such as CF capable of competing with amethopterin might be a process responsible for resistance in antifolics. The conversion of folic acid to CF *in vivo* as demonstrated by Bleiler *et al* (2), Sauberlich (28), Broquist *et al* (6), Girdwood (19) and others and *in vitro* by Nichol (25), Nichol and Welch (26), Broquist *et al* (5), Hendlin *et al* (21), Foley and Haley (18) and others suggested that CF might be the biologically active form of folic acid. Recent studies by Greenberg (20), Kisliuk and Sakami (24), Silverman and Keresztesy (30), Nichol *et al* (27) and Bolinder *et al* (3) suggest that CF may not be the active formylating factor in nature. CF may indeed be the heat stable form of the biologically active factor.

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|---|---|---|---|---|
| | Relative specific activity of PNA | | | |
| *S faecalis* | Adenine | Guanine | Adenine | Guanine |
| O | 48 | 44 | 39 | 41 |
| A | 58 | 65 | 55 | 56 |
| MP | 19 | 4 | 1 | 12 |
| MP/A | 36 | 51 | 39 | 45 |

$$\text{* Relative specific activity} = \frac{\text{counts per minute per mole of isolated compound}}{\text{counts per minute per mole of added compound}} \times 100$$

reasonable to suggest that SF/MP is unable to use adenine and guanine for the biosynthesis of PNA purines SF/MP/A was similar to the parent culture showing a slight preference for the *de novo* pathway

Increased resistance to a drug regardless of the mechanism accompanied by a concurrent increase in sensitivity to another drug would be an ideal system for the chemotherapist This phenomenon has been observed in antibiotic resistance in a number of bacterial studies (32) and in 6 MP and 2 6 DAP resistant *L casei* (13 14) The measurement of the comparative inhibitory effects of amethopterin 2 6 DAP 6 MP and unsubstituted purine on the strains of *S faecalis* (Table VII) show that in SF/A resistance to amethopterin resulted in an increased sensitivity to 6 MP an increased resistance to 2 6 DAP and no change for unsubstituted purine Resistance to 6 MP in SF/MP was accompanied by a slight increase in resistance to amethopterin and 2 6 DAP with no change in respect to unsubstituted purine SF/MP/A by its nature was resistant to 6-MP and amethopterin and since SF/MP/A can use 2 6

enzyme system as that which results from resistance to amethopterin Further studies show that the amounts of enzyme(s) produced by SF/A are considerably greater than those produced by SF/O For example by increasing the substrate concentration of PGA twenty five times there was less than a twofold increase in CF production by SF/O whereas in the same type of experiment SF/A formed about two hundred times as much CF

### Summary

Among these three resistant strains of *S faecalis* a number of quantitative changes have been observed Unfortunately not one is strictly characteristic for a specific mutant Resistance to amethopterin has resulted in a population that is less able to carry out nucleic acid synthesis *de novo* and is less able to use CF than PGA but has an increased capacity for the enzymatic formation of CF It is not surprising that *SF/A has the ability to form more CF since it is less efficient in the use* of CF Perhaps this shift in metabolism tends to compensate for the inefficiency

Resistance to 6-MP has resulted in a population that is unable to use purines with the exception of xanthine and isoguanine for nucleic acid synthesis The capacity for *de novo* biosynthesis of purines is much more efficient than in the parent strain The mutant uses PGA and CF to the same extent and is similar to the amethopterin resistant mutant in respect to the enzymatic formation of CF

The double mutant, SF/MP/A which has the same degree of resistance to 6-MP and amethopterin as the two single mutants quantitatively follows the same pattern as the parent strain (SF/O) in regard to the utilization of exogenously supplied natural purines The growth response to PGA is similar to the parent strain but CF is twice as active as a growth factor The enzymatic formation of CF by SF/MP/A is both more rapid and more efficient than that of any of the strains

Not one of the mutants uses exogenous thymine preferentially to the *de novo* synthesis of nucleic acids With SF/O thymine produces a definite sparing effect on the amount of PGA required

The increased sensitivity of SF/A to 6-MP associated with an increased resistance to 2 6-DAP and no change in response to unsubstituted purine is consistent with the observations (12 15) that these three purine derivatives have different modes of action and shows that even though a mutant has a decreased ability to carry out *de novo* synthesis of purines it is not uniformly more sensitive to several purine analogs The increased resistance of SF/MP to amethopterin with an apparent increase

SF/O formed almost as much CF as SF/A in the 2 hour incubation period but SF/A formed CF at a considerably greater rate CF formation of SF/MP was of the same order of magnitude as CF formation of the other two strains the peak production was reached in the first 20 minutes and after a short plateau period this was followed in time by an

TABLE VIII

EFFECT OF GLUCOSE ON CITROVORUM FACTOR FORMATION

(Each beaker contained a total of 5 ml including 5 mg of cells 5 mg of ascorbic acid 5 2 mg of serine 1 γ of PGA pH 6 5 phosphate buffer and glucose as indicated Incubation at 37 in a Dubnoff incubator )

| Time minutes | Glucose 9 mg | Total (mγ) natural CF formed by | |
|---|---|---|---|
| | | SF/O | SF/A |
| 15 | – | 3 | 105 |
| 15 | + | 4 | 168 |
| 30 | – | 3 | 89 |
| 30 | + | 7 | 141 |
| 120 | – | 7 | 106 |
| 120 | + | 160 | 96 |

TABLE IX

EFFECT OF TIME ON THE FORMATION OF CITROVORUM FACTOR

(Each beaker contained a total of 5 ml including 5 mg of cells 5 mg of ascorbic acid 5 2 mg of serine 9 0 mg of glucose pH 6 5 phosphate buffer and PGA as indicated Incubation at 37 in a Dubnoff incubator )

| Time minutes | PGA 1 γ | Total (mγ) natural CF formed by | | | |
|---|---|---|---|---|---|
| | | SF/O | SF/A | SF/MP | SF/MP/A |
| 0 | + | 3 | 36 | 3 | 12 |
| 10 | + | 9 | 81 | 72 | 340 |
| 20 | + | 21 | 85 | 126 | 373 |
| 30 | + | 38 | 110 | 105 | 288 |
| 60 | + | 66 | 109 | 99 | 137 |
| 120 | + | 151 | 162 | 84 | 98 |
| 120 | – | <2 | <2 | <2 | <2 |

apparent gradual decreased formation of the factor SF/MP/A has characteristics of both SF/MP and SF/A—rapid and very efficient conversion of PGA to CF followed by a decreased formation of the factor after 30 minutes

The results on the formation of CF by the *S faecalis* strains suggest that the resistance to 6 MP imparts a similar effect on the CF forming

14 Elion G B Singer S Hitchings G H Balis M E and Brown G B *J Biol Chem* **202** 647 (1953)
15 Elion G B VanderWerff H Hitchings G H Balis M E Levin D H and Brown G B *J Biol Chem* **200** 7 (1953)
16 Farber S Diamond L K Mercer R D Sylvester R F Wolf J and Lenz C G *Am J Diseases Children* **77** 129 (1949)
17 Flynn, L M., Williams V B O Dell B L and Hogan, A G *Anal Chem* **23** 180 (1951)
18 Foley G E and Haley E C *J Bacteriol* **66** 7_7 (1953)
19 Girdwood R H *Edinburgh Med J* **57** 72 (1950)
20 *Greenberg G R J Am Chem Soc* **76** *1458* (1954)
21 Hendlin D Koditschek L K and Soars M H *J Bacteriol* **65** 466 (1953)
22 Hutchison D J *Ann N Y Acad Sci* **60** 212 (1954)
23 Hutchison D J and Burchenal J H in Chemistry and Biology of Pteridines" J & A Churchill London 1954
24 Kisliuk, R L and Sakami W *J Am Chem Soc* **76** 1456 (1954)
25 Nichol C A *J Pharmacol Exptl Therap* **110** 40 (1954)
26 Nichol C A and Welch A D *Proc Soc Exptl Biol Med* **74** 52 (1950)
27 Nichol C A Zakrzewski S F and Welch A D *Proc Soc Exptl Biol Med* **83** 272 (1953)
28 Sauberlich H E *J Biol Chem* **181** 467 (1949)
29 Sevag M G Reid R D and Reynolds O E eds Origins of Resistance to Toxic Agents" Academic Press New York 1955
30 Silverman M and Keresztesy J C *Federation Proc* **12** 268 (1953)
31 Stokes J L *J Bacteriol* **48** _01 (1944)
32 Szybalski W and Bryson V *J Bacteriol* **64** 45_ (195_)

## General Discussion

Dr Robert Guthrie (Buffalo New York) I should like to mention some results which I think will be of concern to anyone who is interested in unusual sensitivity th t accompanies resistance to antimetabolites Both of the last papers have mentioned examples where resistance to amethopterin results in increased sensitivity to purine antago ists At Roswell Park Memorial Institute recently we have used this princ ple in an attempt to find some interesting agents to put to te t as antitumor agents Spec fically we have isolated a number of mutants of *Bacillus subtilis* that are resistant to amethopterin and a number of mutants of *Escherichia coli* that are resistant to 6-mer aptopurine The results o far have been very encouraging in indicating the usefulness of collateral sensiti ity (increased sensitivity to a tested drug accomp ying increased res stance to another agent) as a method of finding new useful agents Out of approximately eighty various subst tuted purine and pyrimidine analogs including the metabolites as well as a number that we could get from other laboratories subjected to both types of this collateral sensiti ty screening we found

in *de novo* synthesis indicates that amethopterin resistance is not necessarily associated with an increased requirement for exogenous purines

Resistance to both 6-MP and amethopterin appears to have canceled out the changes of the single mutants in regard to synthesis *de novo* and the utilization of exogenous purines which indicates that some other mechanisms may be responsible for the resistance

The results to date on clinical evaluation of the effect of amethopterin on 6 MP resistant leukemias and the effect of 6 MP on amethopterin resistant leukemias showed approximately the same percentage of response in each series as was seen in a previously untreated series of patients (7)

It would be unwise to assume that drug resistance in several mutants will result in the same metabolic alterations The various changes in three drug resistant mutants of *S faecalis* show that a unique route to resistance does not exist Thus the study of the effect of new drugs on bacterial mutants may not lead to a simple solution of the problem of resistance in chemotherapy Drug resistant mutants supply systems that are extremely useful for the elucidation of the multiplicity of processes responsible for cell growth and as such will provide information which will aid in the eventual solution of the problem of resistance to chemotherapeutic agents

REFERENCES


1 Balis M E Levin D H and Luzzati D *J Biol Chem* 216 9 (1955)
2 Bleiler R E Johnson D and Parsons H T *J Nutrition* 56 163 (1955)
3 Bolinder A Widoff E and Ericson L E *Ark v Kemi* 6 487 (1954)
4 Broquist H P Brockman J A Jr Fahrenbach M J Stokstad E L R and Jukes T H *J Nutrition* 47 93 (1952)
5 Broquist H P Kohler A R Hutchison D J and Burchenal J H *J Biol Chem* 202 59 (1953)
6 Broquist H P Stokstad E L R and Jukes T H *J Lab Clin Med* 38 95 (1951)
7 Burchenal J H Ellison R R Murphy M L Karnofsky D A Sykes M P Tan T C Mermann A C Yuceoglu M Myers W P L Krakoff I and Alberstadt N *Ann N Y Acad Sci* 60 359 (1954)
8 Burchenal J H Murphy M L Ellison R R Sykes M P Tan T C Leone L A Karnofsky D A Craver L F Dargeon H W and Rhoads C P *Blood* 8 965 (1953)
9 Burchenal J H Waring G B and Hutchison D J *Proc Soc Exptl Biol Med* 78 311 (1951)
10 Davis B D and Maas W K *Proc Natl Acad Sci U S* 38 775 (1952)
11 Eagle H *J Bacteriol* 68 610 (1954)
12 Elion G B Singer S and Hitchings G H *J Biol Chem* 208 477 (1954)
13 Elion G B Singer S and Hitchings G H *J Biol Chem* 204 35 (1954)

Part IX

# Round Table Discussion on Therapy

## Present Status of New Clinical Agents and Combinations in *Treatment of the Leukemias*

Chairman
**Frank H Bethell**
The Thomas Henry Simpson Memorial Institute for Med cal Research
University of M chigan
Ann Arbor M ch gan

Panel

**Joseph H Burchenal**
Memorial Center for Cancer
and Allied Disease
New York New York

**William Dameshek**
New England Center Hospital
Boston Massachusetts

**Charles A Doan**
Ohio State University
College of Medicine
Columbus Ohio

**Sidney Farber**
The Children s Hospital
Boston Massachusetts

**Alfred Gellhorn**
Columb a University College
of Physicians and Surgeons
New York New York

**Joseph M Hill**
Wadley Research Institute
and Blood Center
Dallas Texas

**Sven Moeschlin**
Buergerspital
Solothurn Switzerland

**Maxwell M Wintrobe**
University of Utah
College of Medicine
Salt Lake City Utah

only two cases of definitive collateral sensitivity The first was that of a very large increase in sensitivity to amethopterin accompanying 6-mercaptopurine resistance in one mutant The other case was a very definite increase in sensitivity to analogs of the pyrimidine moiety of thiamine accompanying amethopterin resistance This has since been extended to five other analogs of the pyrimidine moiety of vitamin $B_1$ They all show an unusual ability to inhibit amethopterin resistant mutants of *Bacillus subtilis*

Further Dr Loebeck in the last two or three months has tested approximately 150 additional purine and pyrimidine analogs and related compounds with two particular mutants one of which is an *E coli* mutant resistant to 6-mercaptopurine and the other is triply resistant to 6 mercaptopurine 6-thioguanine and 8 azaguanine In the second mutant she has found definite collateral sensitivity only in the case of the same antagonists of the vitamin $B_1$ pyrimidine moiety As a consequence of these findings Dr Holland and I tested one of these antagonists 2 methylmercapto-4 amino-5-hydroxymethylpyrimidine in mice and found significant ability to inhibit three different types of tumors At the present time he is investigating the toxicity of this latter compound in dogs What I wish to emphasize is not that we may have perhaps found something of great significance in cancer but that the phenomenon of collateral sensitivity appears to be as specific as the converse phenomenon of cross resistance Of 245 purine and pyrimidine analogs that we have tested so far we have found collateral sensitivity accompanying either 6 mercaptopurine resistance or amethopterin resistance for only two classes of compounds I think this indicates that there is a possibility that new metabolic relationships may be uncovered by a further investigation of this phenomenon

DR GEORGE J FRUHMAN (New York New York) I am unfamiliar with resistance in the leukemia animal and I should like to pose an open question concerning the leukemic animal receiving antimetabolite therapy and then later revealing resistance in the leukemic cells to the antimetabolite In these animals do the normal cell constituents of the bone marrow gastrointestinal tract and so on also reveal the resistance and if so is it to the same degree?

DR CHARLES A NICHOL (New Haven Connecticut) In relation to the question asked regarding resistance occurring in other cells there is rather good evidence that resistance is confined to the leukemic cell because of the manner in which these cells retain this characteristic upon repeated transfer This technique cannot be applied to the other cells which were concerned in this question but I believe one example may be cited in which cells of the intestinal epithelium became refractory to inhibition of respiration by the folic acid antagonists It may be possible that resistance can occur in other cells

In most of these resistant sublines of leukemia in mice a selective process involving a number of transfers achieves a high degree of resistance Whether or not a selective process applies in the case of human patients who become refractory to treatment with these drugs is a question that is technically difficult to answer

## Round Table Discussion on Therapy

DR FRANK H BETHELL, Chairman This evening's round table session has the title "Present Status of New Chemical Agents and Combinations in Treatment of the Leukemias" May I ask your indulgence for a few introductory remarks

In taking up the subject of the therapy of the leukemias especially the acute forms it is unavoidable that we depart to a limited extent from the spirit of complete objectivity that has until now characterized this meeting The attitude of the physician one might say his personal moral philosophy may determine both the form of therapy employed and the energy with which it is pursued

There are those who take a negative position some of them leaders in the profession and distinguished in other fields of medical research. Faced with a case of acute leukemia these physicians follow a program which may be characterized by the terms commiseration palliation, and defeatism I respect their feelings and their motives but I do not agree with them

I believe the members of this panel are positivists One can of course be too aggressive but I know that my colleagues on this platform as well as all other physicians with whom I am acquainted who have such responsibilities are governed by the cardinal rule of the doctor Let your treatment do no harm

We plan to divide this discussion into two major parts The first portion deals with Dr Wintrobe's initial presentation on the criteria for evaluation of results in the therapy of leukemia In dealing with different series of cases of leukemia it is not always possible to apply the criterion of a 50% survival in 30 days or 30 weeks or 30 months We do not always know on what basis the physicians chose the particular patients who were to receive a certain form of treatment

Dr Wintrobe will try to put us on as common a ground as possible in this very difficult field We shall then proceed to a consideration of those leukemias which are generally included in the acute group "Acute" of course is a misnomer because some patients with acute leukemia live longer than do most with chronic leukemia but I think the members of this audience know what we refer to when we talk about "acute" leukemia

Everyone who has seen many cases of acute leukemia will recognize the fact that the cases vary greatly from one to the other in the rate at which they progress Some seem to move on very rapidly in spite of everything we try to do even with the newest and best therapeutic agents Others I suspect take a languid course regardless of what we do

These differences in the cases must be considered in evaluating results Everyone is familiar too with the fact that "spontaneous" remissions occur in a small proportion of cases These seem to occur more often in children than in adults What is their significance? What is their cause? What role do they play in our interpretation of end results?

Everyone I believe will admit that there is a great deal of variation in the responsiveness of children as compared with that of adults I like the Chairman do not consider that there is any hard and fast line at age 13 but that there is a difference in the responsiveness of children one cannot deny What is the reason for that difference? I do not know the reason but it is something we need to give a great deal of attention to if we wish to evaluate our results

I think we should consider the possibility that we may confuse ourselves if we observe the effects of a therapeutic agent in all acute leukemias and do not attempt to differentiate them according to type and perhaps also in relation to other factors If we take the average of all the results our data will be less valuable than they would be if we discriminated between the cases in so far as we are able

This is very difficult to do I certainly am very ready to admit this fact, but I wish to call attention to the need for differentiation In particular I think it is most important that we not assume that all acute leukemias are one and the same

Dr Law and other speakers as well pointed out differences in leukemia in animals in regard to mouse leukemia Likewise in human leukemia there seem to be differences in the course and in the effectiveness of therapy in various types of leukemia—lymphoblastic myeloblastic monocytic

Just a word also about the evaluation of results If one attempts to survey the literature and the reported results of therapy one is greatly disturbed by the difficulty of comparing one person s results with those of another There are various reasons for this Most important among these are differences in the criteria which have been used in reaching conclusions concerning the effects of therapy

Some evaluate therapy on the basis of morbidity—whether or not a

It has seemed advisable to have the initial part of the discussion deal with the recognized acute forms of leukemia especially as leukemia pertains to children and to young adults Personally I feel that there is no sharp dividing line at the age of 13 or 14 years Then we shall take up the problem of dealing with chronic leukemias with emphasis on forms of therapy which have become more recently available We shall invite general discussions after the group of presentations concerned with acute leukemia and again when the chronic leukemias have been dealt with On the other hand we want this to be a give and take affair and if anyone has a point to make or an issue to bring into consideration or a differing opinion perhaps it would be better done immediately rather than to wait until later

I shall now introduce the members of the panel I am sure all of them are known to everyone here At my extreme right is Dr Max Wintrobe On his left in order are Dr Alfred Gellhorn Dr Joseph Hill and Dr Sven Moeschlin At my left are Dr William Dameshek Dr Sidney Farber Dr Joseph Burchenal and Dr Charles Doan

The opening discussion will be by Dr Wintrobe on the subject of criteria for evaluation of response to therapeutic agents in leukemia

DR MAXWELL M WINTROBE I think that those who followed the papers closely yesterday and who thought of their possible application to clinical medicine must have given a good deal of thought to the question of the kind of material we deal with In my remarks I should like to focus attention on two topics—the material with which we deal and the evaluation of the results of therapy

I would differ a little with Dr Bethell s opening remarks although I am sure that I do not differ with his meaning namely that even in the treatment of patients with leukemia objectivity is necessary and is becoming all the more essential One of our difficulties in the study of the use of therapeutic agents in the leukemias has been a certain lack of objectivity which we must correct if we wish to make any headway

First of all regarding the material with which we deal In regard to the chronic leukemias we may ask whether the material is homogeneous By and large cases of chronic myelocytic leukemia are pretty much the same The material is fairly homogeneous Cases of chronic lymphocytic leukemia on the other hand are less homogeneous There is more variation than in the case of chronic myelocytic leukemia in the natural course of the disease its rate of development its rate of progress

Among the acute leukemias there is the possibility of a great deal of confusion and mistaken judgment because the material varies so much

DR SIDNEY FARBER (Boston Massachusetts) We began on January 1 1947 with a program to search for agents chemicals antibiotics hormones and biological materials which might affect the course of acute leukemia *in children particularly as well as other forms of cancer* We have tried from the very beginning to lay down firm objective criteria to be used in evaluating response These terms have been defined as rigidly as possible We are fully aware that within the framework of the term total care" clinical investigation of new agents has very definite restrictions If these are kept in mind I think that objectivity can be obtained within the limits imposed by this kind of study on man

I offer no apology for the fact that this study has been carried out without as objective criteria as are permitted in studies on the mouse or *any laboratory animal*

Just in passing I should like to comment on Dr Wintrobes excellent introduction I should like to suggest that although we should strive always for very strict evaluation by all the objective methods of clinical investigation when an antileukemic agent is found which is truly curative the patient will give us the answer and we will not have to worry about 2 weeks or 4 weeks or the date of onset or minor differences which might occur from study to study

The Chairman has asked me to make a brief comparison of the antipurines and the antifolics I might say that aminopterin and Methotrexate the two *antifolics in most common use* and *6-mercaptopurine* (Purinethol) and thioguanine the antipurines which have been most used are all effective antileukemic agents which work for short periods of time (to be defined in a moment) each producing a complete remission in about 35% of the cases in about 25% more important partial remission in another 20% producing something less than partial remission and in another 20% causing no effect at all on the leukemic process or on the welfare of the patient

That in brief would summarize the two kinds of agents—the antifolics working usually in about 21 days plus or minus 5 days and the antipurines usually working in about 42 days plus or minus 10 days

There is some variation The remissions with the antifolics last on an average 8 months those with the antipurines a little less than that (somewhere around 6 months in our experience) Both agents are toxic Neither kind of agent need be productive of toxicity if rules of good care of patients are observed if the dose in each case is calculated for the individual patient and if the patient is observed constantly

I shall give a summary of our experience with 549 unselected con

remission has been achieved how many remissions have been achieved The definition of the term "remission" varies a great deal in the literature Others adopt the total survival of the patient as the measure of effectiveness Even survival however is difficult to evaluate Where does one begin? At the time of the onset of disease? It is very difficult to know when leukemia begins At the time of the first symptom or the time of the first diagnosis? That too is open to considerable error It depends in part on the perspicacity of the patient and of the physician and on his ability as a physician

Should one consider survival from the beginning of therapy? That has its objections Then we must remember that the effectiveness of therapy may be interrupted by an intercurrent infection which has not been taken care of successfully or by the occurrence of a hemorrhage which may have brought about an abrupt ending

All these factors need to be evaluated in addition to the question of what we consider to be a response What is response? There is a great deal of variation in the literature in the definition of that term Some are willing to accept rather minor differences and call them responses Others wish to have the patient so well that one cannot recognize the disease any more at that point This too needs definition

Again if one combs the literature one sees often that there is selection of material Instead of the data being reported against the author s total experience material is selected Is it selected in favor of the agent being described? Or is it selected be it unconsciously the other way? One does not know It is difficult for anyone to evaluate much of the material that has been published

These are some of the considerations that I think we need to have in mind in discussing the results of chemotherapy or other forms of therapy in the leukemias

Chairman Bethell I certainly agree with what Dr Wintrobe has said I believe it is highly desirable that each individual s experience be controlled by himself by comparable experience with some other type of treatment It has been extremely difficult to compare one s results directly with those of another individual although I believe that collective experiences under uniform conditions of selection and reporting may to a large extent obviate these difficulties

Dr Farber as everyone here knows has had a very early and very wide experience in the treatment of leukemic children with chemical agents I shall ask him to speak next

that leukemia may not be a single entity On the other hand it may be that we go too far if we think of it as many different pathologic processes I believe that perhaps with the exception of the chronic lymphocytic form the various types of leukemia are fairly closely related One argument in support of this concept seems to me to be provided by the observations of Dr Lloyd Law relative to the number of morphologic variants of mouse leukemia that may be produced with the same agents and under more or less the same conditions It would appear that we are dealing with a variety of host relationships to possibly a number of physiologic situations or exogenous agents and certainly the biochemical reactions as well as the morphologic appearances which are associated with these relationships differ Nevertheless a common and unifying factor in leukemia is not excluded by these considerations nor does variability in responsiveness or resistance to therapy necessarily indicate fundamental differences in the nature of these disorders

We shall take up next the subject of combination therapy Dr Burchenal will talk on the treatment of acute leukemia in children with a combination of azaserine and 6-mercaptopurine Combination therapy can be applied to a variety of agents and sometimes quite unrelated and perhaps irrational mixtures may result I think that if we are going to progress by the use of combination therapy we should have some idea why an agent might be of value when given in combination with another and that's what Dr Burchenal is going to tell us about

Dr Joseph H Burchenal I think we would all agree with Dr Farber that the resistance to these various chemotherapeutic agents is of extreme importance We have felt for a long time that the remissions which are obtained in acute leukemia in childhood with 6-mercaptopurine leave much to be desired They are too short and resistance develops all too rapidly—somewhat more rapidly in fact than with the folic acid antagonists For that reason we have been very interested in any forms of combination therapy which might increase the length of these remissions and thus in turn enhance the practical value of 6 mercaptopurine From the theoretical point of view as suggested by Potter (3) we have been particularly interested in any combination of agents which might cause sequential blocking of the same metabolic pathway at different levels Clarke et al (1) using sarcoma 180 in mice as the test system have studied 6-mercaptopurine in combination with various other compounds They have shown that quarter doses of 6 mercaptopurine and quarter doses of azaserine (*o* diazoacetyl l serine) given together are more effective in inhibiting the growth of sarcoma

secutive patients admitted between January 1 1947 and November 15 1955 The survival in all cases was calculated as of February 15 1956 Nine months is the 50% survival for the series as a whole In the period which included the antifolics and ACTH and cortisone 50% of 343 patients were alive at 8 months For the following period when in addition to the antifolics and ACTH and cortisone we had 6 mercapto purine 50% of the 209 patients survived for 11 4 months

The 10% survival figures are very striking In the first period prior to the use of 6 mercaptopurine it was 17½ months For the entire series including those who died 1 hour after admission as well as those in whom there was sufficient time to attempt to obtain a remission the 10% survival period was 21 1 months Finally in the group seen since 6 mercaptopurine became available the 10% survival period was 29 8 months

Of 116 patients who were admitted after the introduction of 6 mercaptopurine but prior to August 1 1954 there was a 50% survival of 9 9 months For the group admitted between August 1 1954 and November 15 1955 when no new agents of any consequence were intro duced the 50% survival period had increased to 14 5 months

*I think it is fair to say that during this recent period we have increased* total care we have used blood rich in platelets frozen platelets and platelet concentrates in the treatment and prevention of hemorrhage we have used more effective methods against infections and there have been other improvements in what we would call "total care I think what we are looking at here is a result which has been obtained by total care which goes beyond the actual effectiveness of the therapeutic compounds themselves

One problem here could be brought up by all of us that of the phenomenon of resistance So far every agent that we have had has met resistance in all patients except one Why this one child who has now survived for 6 years and 7 months did not develop resistance to Metho trexate whereas all the others became resistant is a problem which if solved will make the agents we are using presently very much more effective We have suggested for many years as has Dr Wintrobe to night that when the final story is told it probably will be shown that we are dealing with several different diseases under the term "acute leu kemia" We shall eventually differentiate these biochemically rather than morphologically as we are trying to do at the present time

CHAIRMAN BETHELL Thank you Dr Farber I have only one com ment to make I believe it has been a very salutary thing to emphasize

Presumably azaserine acts to interfere with the amination of this compound by glutamine to form the 4 aminoimidazole riboside. Mercaptopurine on the other hand works at a considerably higher level of this same metabolic pathway to block the conversion of hypoxanthine ribotide into the polynucleotide purines.

Azaserine alone has no practical value in the treatment of acute leukemia in either children or adults. It is true that we have seen two partial remissions in previously untreated children given azaserine with the leukemic cells in the bone marrow decreasing to 70%. Even these very partial remissions were transient, however, lasting only 3 or 4 weeks and relapsing despite continued therapy. When mercaptopurine and azaserine are given in combination the dose is generally 2.5 mg of mercaptopurine and 2.5 mg of azaserine per kilogram. At this dosage most children will develop some mild degree of redness of the tongue, ulcerations of the tongue and buccal mucosa, and in some it may be necessary to cut the dose of the azaserine component in half.

Figure 2 shows the course of a child treated with this combination and it is apparent that the dose of azaserine had to be interrupted for 2 or 3 days many times along the line and then continued at a slightly lower dose. Generally speaking, however, the mercaptopurine component could be continued at the starting dose throughout therapy. We have treated many patients with various types of neoplastic disease with this combination but I should like to discuss with you the responses of a group of twenty nine children with previously untreated leukemia that have been treated with this combination. These were selected cases, most of them not very acutely ill, and this must be borne in mind when evaluating the statistics. Of this group there were six failures, three partial remissions, and twenty complete remissions according to the definition of remission in acute leukemia of the Clinical Studies Panel of the Cancer Chemotherapy National Service Center. Of these twenty patients with complete remissions three have been under treatment with this particular combination for 15, 18, and 22 months respectively, and the average length of time in remission for the whole group was 5.8 months. This is the actual time that they were in remission, which is naturally considerably less than the total time they were under treatment. This length of remission is approximately 2 months longer than the average we have been able to obtain with mercaptopurine. For this reason these results would suggest that when mercaptopurine is given to children with acute leukemia there would be an advantage in the simultaneous administration of azaserine. The control data on

180 than full doses of either compound alone For this reason we have studied a combination of these two drugs in patients with acute leukemia

The theory behind this is shown in Fig 1 which depicts the *de novo*

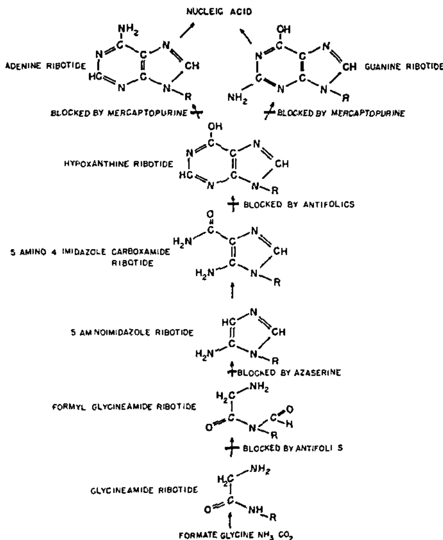



Fig 1

synthesis of nucleic acids from small building blocks such as formate glycine ammonia and CO Both Hartman *et al* (2) and Skipper (personal communication) have shown that the addition of azaserine to such a system causes a pile up of formyl glycineamide ribotide in the medium

mercaptopurine are not completely satisfactory at present, however and for that reason a cooperative study has been set up with several other groups throughout the country to evaluate on a randomized alternate case system mercaptopurine and azaserine versus mercaptopurine alone in previously untreated acute leukemias in children It is hoped that in a year from now it will be possible to state with confidence that this combination is superior to mercaptopurine alone

REFERENCES

1 Clarke D A Philips F S Stock C C Elion G B and Hitchings G H *Abstr 125th Meeting Am Chem Soc* (Kansas City) (March 1954)
2 Hartman S C Levenberg B and Buchanan J M *J Am Chem Soc* 77 501 (1955)
3 Potter V R *Proc Soc Exptl Biol Med* 76 41 (1951)

CHAIRMAN BETHELL I suppose that 99% of patients who receive a diagnosis of acute leukemia are treated with either ACTH or one of the corticosteroids As Dr Burchenal stated in centers where chemotherapy is under evaluation patients who are not considered satisfactory for so called drug treatment ordinarily receive one of these hormones and yet I do not believe we should relegate endocrine therapy to a last resort or to the status of mere palliation

What do these agents actually accomplish? Are there advantages in using unusually large doses? Dr Joseph Hill probably was the first to employ high dosage corticosteroid therapy in the treatment of acute leukemia Dr Alfred Gellhorn also has had quite a wide experience in this field We shall hear from Dr Gellhorn later Now I should like to call on Dr Hill

DR JOSEPH M HILL In assessing the effects of massive steroid therapy I should like to consider this treatment against the background of our results in 121 cases of acute leukemia I should like to call your attention to the remission rate in two divisions containing approximately equal numbers of cases first those which had available as therapy antifolics ACTH and cortisone and second those which had mercaptopurine azaserine and massive steroids

*Survival time has increased greatly* with an increase in the over all figure from 12% in the first period to 42% surviving beyond 12 months in the second period The remission rate at the same time increased from 44% to 83% Only complete or good partial remissions based on laboratory findings were included

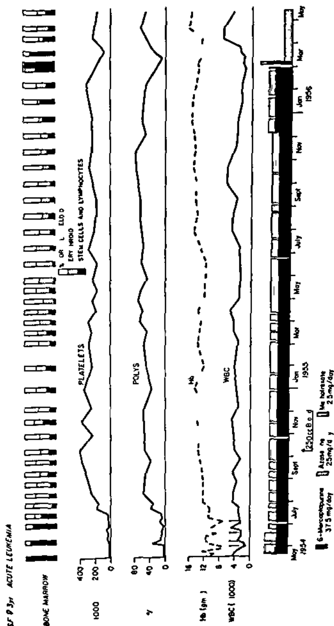

SF ♀ 3 yr ACUTE LEUKEMIA
BONE MARROW
1000
400
200
0
PLATELETS
%
80
40
0
POLYS
Hb (gm)
16
12
8
4
Hb
WBC (1000)
4
0
WBC
GR
L
ERY MROID
ELLOD
STEM CELLS AND LYMPHOCYTES
May 1954
July
Sept
Nov
Jan 1955
Mar
May
July
Sept
Nov
Jan 1956
Mar
May
6-Mercaptopurine 37.5 mg/day
Azaserine 25 mg/day
Na folinate 2.5 mg/day
250 cc B.o.d.

FIG. 2

however to three monocytic leukemias two shown in the table and one observed since then Two complete remissions and one partial remission were obtained One of these was a lady 81 years old and the other two were also adults

TABLE II

RESULT OF TREATMENT ON PATIENTS RECEIVING PREDNISONE AND PREDNISOLONE

| Type of leukemia | Failure | | Partial remission | | Complete remission | | Total |
|---|---|---|---|---|---|---|---|
| | A N | ♂ 35 † | F H | ♂ 47 | T R | ♂ 62 | |
| Acute | C G | ♂ 69 | J S | ♂ 17 | R S | ♂ 8 | |
| granulocytic | E R | 41 † | E C | ♀ 22 † | | | |
| | | | E B | ♂ 51 | | | 10 |
| | K L | ♀ 41 | J E | ♂ 4½ | J G | ♂ 12 † | |
| | J M | ♀ 20 | | | K B | ♀ 3 | |
| Acute | S H | ♂ 30 † | | | J M | ♂ 12 | |
| lymphocytic | | | | | C H | ♂ 1 | |
| | | | | | E R | ♂ 26 † | |
| | | | | | M G | ♂ 11 | |
| | | | | | M E | ♂ 8 | |
| | | | | | N M | ♀ 51 | 11 |
| Acute | | | M H | ♀ 81 † | A H | ♀ 34 | |
| monocytic | | | | | | | 2 |
| **TOTAL** | 6 | | 6 | | 11 | | 23 |

Leukemia previously not treated
† Steroid only specific chemotherapy

A few brief additional cases may be of interest Prednisolone was given in doses of 3 g daily in a case of acute monocytic leukemia and a complete remission was obtained Initially the bone marrow of this patient was rather hypoplastic containing cells that might be termed monoblasts or monocytic reticular cells Two weeks later the bone marrow was regenerating with normal cells

As an example of mixed therapy an initial remission was obtained in this case with 3 g daily of hydrocortisone This woman was 4½ months pregnant and it was very desirable to try to get her into remission and to use as little of the antimetabolites as possible for fear of adverse effect on the pregnancy There was an abrupt rise in the platelets coincident with the completion of therapy with the massive doses of steroid At this time amethopterin was started and the dosage was kept as low as possible A little later mercaptopurine was

When we first undertook this work we tried hydrocortisone therapy in a few cases giving up to about 3 g /day and then 9 α fluorohydrocortisone a much more potent compound became available and this was used In Table I is summarized our somewhat earlier experience

TABLE I
RESULT OF TREATMENT ON PATIENTS RECEIVING MORE THAN 250 MG OF 9 α FLUOROHYDROCORTISONE (18 CASES)

| Type of leukemia | Failure | Partial remission | Complete remission | Total |
|---|---|---|---|---|
| Acute myeloblastic | F W ♂ 24<br>L F ♀ 47<br>J S ♂ 45 | R B ♂ 2<br>*D P* ♀ *66*<br>J G ♀ 40 | D J ♀ 14 †<br>*J F* ♂ 33<br>J S ♂ 17 †<br>S W ♂ 45 †<br>Z E ♀ 20 † | 11 |
| Acute lymphoblastic | S J ♀ 7<br>K L ♀ 4' | J R ♀ 7<br>Terminal phase | J S ♂ 7<br>B V ♂ 2<br>R L ♂ 17 | 6 |
| Plasma cell | | | F R ♀ 66 † | 1 |
| TOTAL | 5 | 4 | 9 | 18 |

Leukemia previously not treated
† FHC only specific chemotherapy

with this compound You see that a fairly good remission rate was obtained In this particular group we had the division into failure partial remission and complete remission as shown Among the patients in this group were a larger number of cases of acute granulocytic leukemia than usual In the case of the rather rare plasma cell leukemia there was a most gratifying response to the 9 α fluorohydrocortisone with the clearing of the plasma cells from the peripheral blood and from the bone marrow in approximately 9 days time The patient had a remission of about 2 months and thereafter nothing was of any avail In this case we gave doses up to 200 mg /day of 9 α fluorohydrocortisone which has a potency twenty five times that of hydrocortisone

Sometimes a remission can be obtained with exceeding rapidity after fluorohydrocortisone in massive doses In one such instance 200 mg /day was given and at the end of 3 days the blasts were cleared from the peripheral blood and reduced in the bone marrow below 10% in 9 days

The series treated with prednisone and prednisolone (Table II) contained more lymphocytic than granulocytic cases Attention is called

TABLE III
SIDE EFFECTS AFTER TREATMENT WITH PREDNISONE AND PREDNISOLONE

| Name age sex | Abnormal | | Blood sugar | | Blood pressure rise | | | | Psychosis depression euphoria | Weight gain over 5 lb | Excessive weakness or low blood pressure on withdrawal |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Systolic | | Diastolic | | | | |
| | K | Na | 1.0-160 | Over 160 | 10-20 | Over 20 | 5-10 | Over 10 | | | |
| N M 51 F | | L | | Yes | | Yes | | Yes | Yes | | |
| M H 80 F | L | | Yes | | | | | | | | Yes |
| T R 6_ M | | | | Yes | | | | | | — | Yes |
| S H 30 M | | | | Yes | | | | | — | | — |
| E B 52 M | L | | Yes | | | | | | Yes | | — |
| F H 47 M | | | | Yes | | | | | | | |
| C H 44 M | — | — | — | — | — | — | — | — | | Yes | |
| A N 35 M | H | | | Yes | | | | | | | |
| O S 46 M | | | Yes | | — | — | — | — | | Yes | |
| R H 44 M | H | | | Yes | | | | | | Yes | |
| O H 34 F | — | — | — | — | | | | | | | |
| N M 74 M | | | | | | Yes | | Yes | | Yes | |
| B S 53 F | — | — | — | — | Yes | | | Yes | | Yes | Yes |
| E R 41 M | | L | | | | Yes | | Yes | | | |
| T S 72 M | | | — | — | | | | | | | |
| C V 56 M | | | | | | | | | | Yes | |
| C G 70 M | | | | Yes | | | | | | — | |
| Total | 4 | 2 | 3 | 7 | 1 | 3 | 0 | 4 | 2 | 6 | 3 |

L = below normal range H = above normal range — = not determined blank space = normal values

also employed Again small doses of prednisone were given and finally some massive doses of prednisone were used The pregnancy was successfully completed and a healthy baby was obtained but the mother died of a ruptured spleen 6 weeks after delivery

The chief adverse effect of fluorohydrocortisone is loss of potassium requiring doses as high as 100 to 110 meq of potassium a day to maintain balance With adequate potassium and low sodium diet normal sodium and potassium levels in the blood have been maintained however There was one case of psychosis in the fluorohydrocortisone series that was quickly reversible

Table III summarizes some of the side effects with prednisone and prednisolone In this series there was relatively little difficulty with sodium and potassium although there was some deviation from normal levels because the patients were not watched as closely as in the other series The blood sugar rose in a great many cases but in no instance was hyperglycemia a contraindication to the use of the drug and we did not discontinue it for that purpose There was some effect on elevation of blood pressure in no instance requiring discontinuance of the drug Psychoses occurred in two cases in one of which convulsant therapy was required The patient is now doing well and is having a fine remission Weight gain due to retention of water presented some difficulty By and large however this was not a serious problem The patients were watched carefully and the steroid was discontinued or the dosage lowered for a while if necessary Weakness on withdrawal of the drug was something of a problem with more difficulty in older patients We followed the policy of tapering off the drug and giving increasing and then decreasing doses of ACTH

The cytolytic effect of the therapy on the leukemic cells and the rapidity with which it can occur with these massive doses of steroid was manifest in a case of acute myelogenous leukemia A few days after institution of treatment a change was apparent in the leukemic cells of the marrow involving some breaking up of the cells The disintegration was progressive and ultimately almost complete The bone marrow then underwent regeneration and complete remission was obtained As in most of these cases however the remission was not long They have ranged from about 6 weeks to 8 weeks and a little over

It may be worth while to review briefly our experience with high dosage corticosteroid therapy in the chronic leukemias The results of such treatment in chronic lymphocytic leukemia are rather interesting

An 81 year old man with chronic lymphocytic leukemia was treated with massive doses of steroids This individual had exceedingly large

CHAIRMAN BETHELL We shall defer discussion on the treatment of chronic leukemia with the steroids because I know Dr Dameshek and others have prepared comments to make on this topic We are now at the dividing point in our panel discussion so I will ask each member if he wishes to comment on some phase of the treatment of acute leukemia. First I should like to ask Dr Gellhorn to speak on the subject of massive corticosteroid therapy in acute leukemia with special reference to adults

DR ALFRED GELLHORN I should like to compare and contrast the results obtained by Dr Helen Ranney and me with those presented by Dr Hill. Our experience is much more limited than that of Dr Hill we have evaluated the effect of large doses of either cortisone or prednisone alone rather than in combination on acute leukemia

We have attempted in this study to limit ourselves to patients who are past the teen age We have three patients however two teenagers and one child of 8 who had become refractory to all other forms of therapy who also received large doses of prednisone I suppose the rest are in the classification of adults being 28 years of age or older

Before I show you our results I should like to state that our experience has not been quite the same as Dr Hill's in terms of toxicity We have developed a very real respect for massive doses of steroids and our toxicities include such minor things as the rapid development of moon facies euphoria and occasionally psychotic manifestations One of our patients was so convincing that he almost had me buying into his business

The problem of steroid diabetes in our experience also is not minor for when acidosis develops it presents difficulties in management

We have had two individuals who have had perforation of a viscus The problem of salt and water retention as indicated by Dr Hill is a real one in some individuals and that of potassium loss is also important and must be carefully guarded against

Finally in a number of our patients the problem of infection constitutes a serious complication In several instances septicemia which could not be controlled by antibiotics was the cause of death

Table I summarizes our results We have sixteen patients who fall into the category of easily diagnosed acute leukemia All patients who were treated with the exception of one single patient had acute symptoms These were not in the group that Dr Wintrobe has mentioned—patients with acute leukemia who at the moment are not symptomatic All these patients were symptomatic

lymph nodes which in the fruit and nut system of measurements reached the size of lemons in the neck and almost the size of oranges in the axilla *We were very much interested to see what massive steroid would do to the tumors* In this case we gave the medication and then decreased the dose slightly then resumed full dosage dropped down and again gave the full dose Because of the patient's age and because he was retaining water we thought it unwise to continue and changed to a more standard form of therapy namely radioactive phosphorus A lymph node puncture prior to therapy revealed small *lymphocytes* rather uniform in their staining and nuclear characteristics Soon after treatment two kinds of cells were present—those that had spread out and stained more lightly and darker staining cells very similar to those *seen before therapy* Later there was progressive lightening spreading out of the nuclei and apparent lysis of the cells The lymph node masses *regressed rapidly but the count* stayed high so we tried it again and with the next case we were more successful

We were a little more persistent in treating our next patient a man aged 72 with chronic lymphocytic leukemia and in this case we not only obtained regression of lymph nodes but also recession of the count to normal The percentage of lymphocytes likewise returned to normal It would have been difficult to have made a diagnosis of leukemia during this period of remission

To summarize we have used the steroids in massive dosage in a total of fifty six cases of leukemia and allied conditions Of these forty five were acute leukemia Part of this group received steroids without prior specific therapy and I should like to analyze these a little further In this group of sixteen cases there were twelve that showed remissions and four that did not All the remissions were complete except one a good partial giving an over all remission rate of 75% In so far as possible massive steroid therapy was given to consecutive cases During this period however there were ten cases in which this type of steroid therapy was considered contraindicated because of possible danger Further analysis showed that there were eleven cases over 21 years of age with six complete and one partial remission and four with no remission

There were in addition twenty nine cases with mixed therapy and many of these were cases in which the steroid was used late in the disease when prior therapy had become ineffective Of those above 21 years of age there were six that showed no remission and seven that did In those under 21 years of age we had six showing no remission and ten that did show a remission

TABLE I (*Continued*)

| Patient | Age | Sex | Diagnosis | Duration of symptoms before steroid therapy | Previous therapy | Effect | Comment | Current status (months after first course of massive steroid) |
|---|---|---|---|---|---|---|---|---|
| S R | 13 | M | Acute lymphatic leukemia | 2 months | None | Complete remission | Two further complete remissions | Living (7 months) |
| M R | 27 | F | Acute myeloid leukemia | 1 month | Prednisone 75 mg o d | Partial remission | | Living (5 months) |
| M F | [illegible] | F | Acute myeloid leukemia | 4 months | Prednisone 75 mg o d | Complete remission | | Living (5 months) |
| L F | 69 | F | Acute leukemia ? monocytic | 5 months | Transfusions | None | | Dead (1 month) |
| N deS | 32 | M | Acute lymphatic leukemia | 6 weeks | Prednisone 75 mg o d | Partial remission | | Dead (2 months) |
| A I | [illegible] | M | Acute lymphatic leukemia | 2 weeks | Prednisone 75 mg o d | Complete remission | | Living (3 months) |
| M G | 33 | F | Acute lymphatic leukemia | 4 weeks | Transfusions prednisone 75 mg o d | Partial remission | Pregnant | Living (6 months) |
| W F | 14 | M | Acute myeloid leukemia | [illegible] months | 6-MP | Partial remission | | Living Lost to follow up (3 months) |

TABLE I
EFFECT OF PREDNISONE IN ACUTE LEUKEMIA (1000 MG DAILY)

| Patient | Age | Sex | Diagnosis | Duration of symptoms before steroid therapy | Previous therapy | Effect | Comment | Current status (months after first course of massive steroid) |
|---|---|---|---|---|---|---|---|---|
| D O B | 6 | M | Acute leukemia | 17 months | Cortisone 6 MP | None | Died on 8th day of steroid therapy | Dead ( 1 month ) |
| M K | 28 | F | Acute myeloid leukemia | 3 months | Transfusions | Partial remission | Diabetes (transient) | Dead ( 5 months) |
| I S | 58 | M | Acute leukemia | 12 months | None | None—? acceleration of disease | Hypomania | Dead ( 1 month ) |
| R K | 40 | M | Acute leukemia | 1 month | None | Complete remission | Subsequently two further complete remissions | Dead (12 months) |
| M C | 33 | F | Acute myeloid leukemia | 3 months | Transfusions | None | | Dead ( 1 month ) |
| I M | 42 | M | Acute leukemia | 10 days | None | Complete remission | Subsequently two more complete remissions | Dead ( 5 months) |
| A I | 42 | F | Acute leukemia | 5 months | 6 MP x ray transfusions cortisone | None | | Dead ( 1 month ) |
| J C | 29 | M | Acute leukemia ? monocytic | 2 months | Hydrocortisone | Partial remission | Infection upper abdominal pain | Lost to follow up (2 months) |
| L Y | 26 | F | Acute myeloid leukemia | 3 months | Cortisone | None | | Dead ( 1 month ) |
| B C | 70 | M | Acute myeloid leukemia | 3 weeks | None | None | | Dead ( 1 month ) |

DR. CHARLES A. DOAN We have tried it both ways during the last ten years in our Hematology Leukemia Clinic and we have very definitely the impression now that we have these newer agents that the relapses come much more quickly and more surely if we do not keep up maintenance therapy.

We have discussed the matter with intelligent patients and we have had in our longer remissions (up to two to three years) individuals who on Purinethol (6-mercaptopurine) for example have been kept on doses as small as 25 mg twice a week. We have not gone below that dose in the last three years.

I have been unwilling to stop the medication when the individual is in complete remission with normal bone marrow and a normal blood picture in health and as some of our young people are in school.

Formerly we did interrupt the therapy only when we could find no evidence of leukemia cells either in bone marrow or in the blood but the longest remission we have had on no therapy at all was 6 months. We have had remissions lasting 12 to 24 months in nearly identical cases on maintenance therapy. We believe at the moment at least in keeping doses small. Our patients in longest remissions are now receiving 6-mercaptopurine 25 mg twice a week.

DR. WILLIAM DAMESHEK Our impression is the same as that of Dr. Doan. We have used maintenance therapy consistently and believe that it effectively controls the leukemic process for a longer period of time than without maintenance therapy.

CHAIRMAN BETHELL The only comment I have to make in this respect is that I think there is a difference perhaps between such agents as Myleran and Colcemid and the mustard group on the one hand, and the antimetabolites on the other because I have the impression that if one gives a metabolic antagonist in under dosage one might as well give nothing as it has no effect at all.

I think Dr. Wintrobe has been rather left out. He introduced the whole subject but didn't have a chance to talk about anything specific.

DR. WINTROBE I have only two or three questions to ask. One question that has run through my mind in relation to massive dose steroid therapy is this. What evidence is there that such large amounts of steroids are doing a job which the more customary doses would not do? I should like to hear the answer to that spelled out as clearly as possible.

I should like to ask Dr. Gellhorn whether he has a breakdown of his

In the group of sixteen patients four went into complete hematological and clinical remission four had a partial effect—that is the hemato logical picture had not been completely converted to normal. There were eight who had no effect whatsoever There were three patients with subacute leukemia with a partial remission in one of these in dividuals and three individuals with the blastic crisis of chronic myeloid leukemia one of whom had a partial remission but only for a few weeks

Our observations in the use of massive doses of steroids started in February 1955 At the present time the results in the individuals who have had a remission are that one person lived for a period of 12 months and died in February 1956 The life span of those patients who have had "complete" or "partial" remissions of their acute leukemia has been from 3 to 12 months however some patients in this group are still alive

I should like to state that we have the impression that in one individ ual who was not clinically acutely ill associated with steroid therapy there was a very abrupt and rapid acceleration of the disease leading to his death We do not know whether this was cause or effect Temporally the relationship was impressive

In those individuals who went into remission it was our observation that the characteristics were heralded by a sharp drop in the peripheral leukocyte count down to leukopenic levels In those individuals who showed a rise in the peripheral white count invariably there was no effect

CHAIRMAN BETHELL Dr Moeschlin do you have some comments you would like to make at this time?

DR SVEN MOESCHLIN I do not know whether we should bring up this point now but I might ask a question here

If a patient with acute leukemia goes into remission shall we then continue with a maintenance dose for the treatment or shall we stop the treatment until the patient has a recurrence of his disease?

Our experience is too limited in children and probably also in adults but we have the impression that the patient goes on better if we keep him on a maintenance dose after the occurrence of a remission I should like to know how the other hematologists and clinicians here feel about this point.

CHAIRMAN BETHELL Probably everyone here has his own opinion but I should like to ask Dr Doan first to give his

places to use them One is the case that can't wait another is the case that won't turn the corner the third is the terminal case where one is trying to get one more remission

CHAIRMAN BETHELL Dr Gellhorn do you have any comments?

DR GELLHORN Dr Wintrobe asked about our breakdown of the sixteen patients We had six myeloid and four lymphatic and six that we could not classify We call them "stem cell" leukemia In terms of remissions in the myeloid cases three had remissions in the lymphatic cases there were four and in the stem cell cases there were two

I want to ask Dr Hill about the duration of the remissions that he has achieved Are these long term remissions?

DR. HILL No the remissions are not long They are short term remissions and therefore the policy is to obtain the remission with massive doses of steroids and as quickly as possible transfer to the antimetabolites One usually has about 2 months in which to do this

The chief virtue of the steroid therapy is speed of action and perhaps a little more certainty in obtaining the remission an attempt is then made to hold it with the antimetabolites

We make no effort after initially finding we are "short" to carry the patients In fact we like to use massive doses and get out as quickly as possible feeling that perhaps resistance does not develop as rapidly that way

CHAIRMAN BETHELL We shall proceed to the question Dr Wintrobe asked Dr Doan about 6 mercaptopurine maintenance dosage

DR. DOAN I agree that the question always comes up as to what is a minimum dose I am not a homeopathic physician but nevertheless there is good reason for giving small doses sometimes and I realize I am on the opposite end of this very high dosage affair

We usually start out with between 100 and 200 mg of 6 mercaptopurine (Purinethol) a day When and as a remission occurs we keep the patient on his optimum dosage or the dosage on which he has gained his remission for 1 to 2 weeks after the remission is established in terms of return to normal of the blood and bone marrow and then we begin to drop the dosage slowly 25 mg /day for a week at a time So we gradually reduce the dosage by about 25 mg /day until we get down to 25 mg arbitrarily (I admit) every 72 hours

In the past, when we have gone to no therapy at all we have had relapses in 6 weeks to 4 or 5 months whereas we have individuals going

sixteen cases of acute leukemia treated with massive doses of corticosteroids

The third question would be to Dr Doan What does Dr Doan visualize 25 mg of 6 mercaptopurine twice a week does? Is it really doing anything? I find it hard to visualize any effect from such a very small dose of an antipurine I ask these questions for information

CHAIRMAN BETHELL Dr Hill can you answer Dr Wintrobe s question? What do these massive dosages accomplish that the more generally used dosages would not do?

DR JOSEPH M HILL I can try I don t know whether I can satisfy him or not

First of all one can lyse cells according to the evidence presented this afternoon That isn t done with the ordinary dosages

I should like to say further that I believe one can obtain a higher percentage of remissions with massive doses particularly in acute myelogenous and monocytic leukemias than have been heretofore reported

I also should like to indicate that in using massive doses of steroids I think one has to do as is done with the antimetabolites one must go ahead and skate on thin ice and utilize dosages that approach toxic levels in order to get the maximum number of remissions I don t think this means 500 mg /day or 1 g /day or any particular dose I believe we have been approaching toxic doses in the range of 3 to 5 g /day of the prednisolone type of compound and 500 to 600 mg /day of 9 a fluoro hydrocortisone These doses are used very cautiously The patients are watched closely and daily counts are done

I have before me a breakdown of cases Of nine untreated myelogenous and monocytic leukemias six had remissions Of seven lymphatic and plasma cell leukemias six had remissions As I stated before all remissions were complete except one myelogenous leukemia

In the group that was mixed in respect to therapy—and this means essentially those cases where other things have failed and when one is getting on toward the end of therapy—of those above 21 years of age we had thirteen cases seven of which obtained remissions These of course were remissions in varying degree We do not have the final figures for the partial remissions as yet None of these is merely a clinical remission We accept only cases having some substantial laboratory evidence of remission

I think there are certain indications for the use of these steroids that have to be considered too in interpreting these figures There are three

When one sees a patient with chronic leukemia, therefore one feels a great deal better Here the condition seems relatively benign certainly in contrast with acute leukemia, and most of the time we can do something about it.

We began to get rather good results in chronic leukemia some years ago with the introduction by the Memorial group in New York of triethylenemelamine (TEM) and I think we can say pretty definitely that we get remissions in approximately 50% of the cases of chronic lymphocytic leukemia that we treat. Of course we don t treat all cases of chronic lymphocytic leukemia. I think it should be realized that in asymptomatic cases occurring in the older age group with counts ranging from 25 000 to 100 000 or so treatment is best withheld Therapy often makes the situation worse and frequently one regrets ever having embarked on a therapeutic course Such individuals are best watched at infrequent intervals if too many blood counts are done the ultimate neurosis may become worse than the disease In the more aggressive cases of chronic lymphocytic leukemia therapy with TEM has been effective in approximately 50% of our cases These cases are certainly not all alike There may well be a metabolic difference between them as indicated by the failure of some cases to respond to TEM and their ready response to CB 1348

As for Myleran, which we have used in chronic granulocytic leukemia, that is about the best of the chemotherapeutic agents that has thus far been introduced Here both the course of the disease and the results with the chemical, which seems to be well nigh specific for this disease are highly consistent. In our series of thirty four consecutive cases of chronic granulocytic leukemia treated with this chemical remissions were induced in practically every instance Our dosages have been rather high particularly when the white count was over 200 000 and we have used doses of 8 to 16 mg / day Within 4 weeks after the initiation of therapy with Myleran the white count is down to levels of approximately 20 000 from initial levels of anywhere from 100 000 to 800 000 We try to get the white count down to relatively low levels i e 5000 and 6000 by persistent maintenance therapy We find that we obtain our best and longest remissions by reducing the leukocyte count down to these levels When this has been accomplished, the drug is discontinued. I realize that at this point one is treading on thin ice and that the white count may then drop to 2000 to 3000 but thus far we have seen no deleterious effects from this procedure The ensuing remissions are often very lengthy Thus in two cases one has gone along for 19

for 2 years on 25 mg twice a week. That is the reason—by trial and error. We try employing the general principle in medicine to give the least medication that will keep an individual in equilibrium. The moment there is some sign of relapse we start to increase the dose and try to keep ahead of the disease. That is the plan of dosage that we have used.

CHAIRMAN BETHELL. I should like to ask Dr Farber if he has any further remarks he wishes to make at this time.

DR FARBER. I should like to make one point about spontaneous remission in acute leukemia. I refer to Dr Diamond's experience prior to 1947 when about 9% of some 300 children with acute leukemia had spontaneous remissions; the average duration was 10 weeks. In discussing the results obtained with newer methods of therapy we are not talking about anything that can be confused with these spontaneous remissions.

CHAIRMAN BETHELL. That is certainly true.

During the past two days I have had an opportunity to talk to members of this panel and others with particular reference to the treatment of chronic leukemias and the place of drug therapy. It seemed at first to be the view of everyone to whom I spoke that we all have pretty much the same ideas; we use the same drugs for the same reasons. But after I had a little more experience and got down to cases and reported back what I had heard, such comments were frequently made as "What? Is that drug actually used for such a type of case and under those conditions?" So I believe there is more reason for discussing the treatment of chronic leukemias than might appear at first glance.

We shall begin the second portion of this program by asking Dr William Dameshek to speak on the general subject of the treatment of chronic leukemia with especial reference to newer agents.

DR DAMESHEK. I think it is a welcome change to get away from the acute leukemias, despite all the therapeutic benefits we have been hearing about. After awhile, particularly when one deals with adults as I do largely, one becomes depressed with the ineffectiveness of our present modes of therapy. This I have called "of pathetic benefit" because if we had really good agents we would throw all our present ones out of the window and would not quibble about relatively minor things as amount of dosage or whether maintenance should be used. We must admit that at the moment our therapeutic efforts, particularly in the adults, are hopelessly inadequate.

and CB 1348 in the lymphocytic variety and the steroids in the auto immune complication of chronic lymphocytic leukemia Hodgkins disease and lymphosarcomatosis

CHAIRMAN BETHELL I think the last point made is a very important one that the dosage of corticosteroid for control of the auto immune process may be very much lower than that which will affect rate of pro liferation or produce lysis of leukemic lymphocytes

We shall now proceed to a discussion of a different type of compound *I dont know who first used colchicine in the treatment of leukemia or other forms of malignant disease I suppose the idea occurred to a number of people after the demonstration of its effect on mitosis in plant cells Those early efforts seemed to come to nothing largely I suppose* because of the toxic side effects that prevented adequate trial of the compound

With the introduction of the analog of colchicine in which a methyl group is substituted for the acetyl group the disadvantages of the parent compound for prolonged therapy have been largely eliminated We have asked Dr Sven Moeschlin to relate to us his experiences with this new drug known as Colcemid or Demecolcine which he first intro duced into the treatment of leukemia

DR MOESCHLIN *First I should like to make some comments con cerning the treatment of the chronic leukemias before I go into the matter of colchicine*

I think it is *very essential to stress the point as emphasized by Dr Dameshek* that the chronic lymphocytic leukemias should not be treated as early as possible The unfortunate thing in Switzerland is that all these chronic lymphocytic leukemia cases come to the radiologist and he just pulls them down with x ray until all the glands and the spleen have disappeared I dont think the patient feels any better at all We also believe that it is beneficial to wait until anemia develops or until the patient is clinically sick

As to the use of prednisone as an antihemolytic agent we have em ployed very small doses the maintenance dose usually being 10 to 15 mg daily and most of the patients have done very well

I also should like to point out that some people believe it is dangerous to treat with TEM patients who have an infiltration of bone marrow with lymphocytes and a leukopenia and thrombocytopenia From the ex perience of many investigators as well as ourselves this is one of the indications for TEM One must give very small doses We usually start

months and *another has gone along for 15 months* without further therapy

Two cases in the series of Myleran treated cases developed thrombo cytopenia and for that reason the drug had to be discontinued A myeloblast crisis developed within 6 months in two cases Whether this was initiated by the drug or came on coincidentally is of course un certain since at least eight of ten of our cases of chronic granulocytic leukemia develop myeloblast crisis as a terminal affair whatever therapy is given *It is our impression based on these cases that Myleran does* not hasten the development of blast crisis unfortunately it does not prevent it

In most cases except those in which the white count has gone down to very low levels we use a maintenance dose of approximately 2 mg daily This simple medication—a pill—seems far better in this disease than x ray therapy it is easier to use it has remarkable specificity and it requires no referral to another specialist

*Now about steroids and chronic leukemia* When in doubt about what to use we use steroids for almost any case of leukemia so we use steroids a good deal perhaps to control bleeding and perhaps to make the patient feel better Sometimes it is a good idea to make the patient feel better rather than to do anything drastic

I am not so sure whether 1000 mg of prednisone or 500 mg or 200 mg represents the correct dosage These massive doses may at times be effective but they certainly are apt to result in very severe fulminating *infections diabetic reactions and all the side effects that Dr Gellhorn* has mentioned—and we must therefore use them with caution

The greatest value of the steroids in our experience has been in chronic lymphocytic leukemia with complicating auto immune hemo lytic manifestation in which their action is almost specific This compli cation develops in approximately one case out of ten The increased hemolysis can be quickly controlled in almost every case with sufficient doses of ACTH or steroids This works out far better than splenectomy in most cases control continues for a long while or until the leukemic process becomes exacerbated In cases of both chronic lymphocytic leu kemia and Hodgkin s disease the hemolytic process may be controlled *by the steroid* even though the underlying proliferative process is not greatly affected by therapy Maintenance therapy with steroid is re quired lest the hemolytic process again relapses

Thus we now *have available for the treatment of chronic leukemia* two or three effective agents Myleran in the granulocytic type TEM

probably the best animal for the evaluation of the inhibitory effect of new cytostatics on granulocytopoiesis So one can say that the leukocytes of men and cats behave similarly It has long been known that the characters of cats and some humans have much in common

Schar *et al* (52) demonstrated an antimitotic action similar to that of colchicine in cultures of normal and malignant cells and in implanted tumors They also showed that the lethal dose i v for mice was thirty to forty times as high as that for colchicine

In our study of the action of Demecolcine on the bone marrow and peripheral blood rabbits and cats were used (44 45)

After preliminary experiments the cats were given 0 25 mg /kg body weight daily intraperitoneally and the rabbits 3 mg /kg intravenously This dosage was chosen to produce a definite change in the blood picture without causing any significant side effects Erythrocytes reticulocytes and leukocytes were counted in all the animals differential counts were prepared and periodic marrow punctures performed

Only the main findings will be indicated here For more details the reader is referred to the dissertation by Lichtman (34)

Two days after the intravenous injection of Demecolcine the total number of leukocytes began to drop owing to a decrease in the neutrophils The effect was delayed about 12 to 14 days in animals treated orally After cessation of therapy the neutrophil count began to rise in 2 days attaining its initial value within 8 to 10 days With the dosage used the lymphocytes were found to be only slightly affected in the cat and negligibly in the rabbit with similar results in the thrombocyte erythrocyte and reticulocyte counts Examination of the bone marrow revealed a definite diminution to approximately one third of the initial values of the myelogenous elements so that the erythropoietic portion appeared relatively increased. During the administration of this rather high dose no side effects were observed in the rabbit other than a definite weight loss and an inhibitory effect on spermatogenesis The cats were more sensitive and were unable to tolerate a dosage of 0 5 mg /kg body weight showing emesis ptyalism muscular pain and difficulty in swallowing No more severe changes were seen in the bone marrow of cats than in the rabbits this is in contrast to our findings in cats given urethan (43)

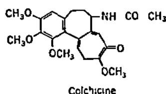
*In summary the doses used namely 0 25 mg /kg in cats and 3 mg /kg in rabbits produced a definite almost elective inhibition of the granulocytopoiesis without any important side effects*

with 2 5 mg and wait 3 weeks if the patient does not get better we give him 5 mg the next time We have seen many remissions with this treatment

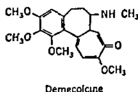
Now I should like to speak about Colcemid Perhaps I am not being completely objective about this because one always likes ones own children first

The purpose of this presentation is to give an account of our present knowledge of the clinical and experimental use of Colcemid and to compare the results with those of other cytostatic substances

Since the work of Jacoby (27) Dustin (19) and Bucher (10) the inhibitory action of colchicine on mitosis with blocking in the meta phase has been well known With the exception of local application to skin tumors its clinical use by Landolt (31) in the therapy of tumors and leukemias failed owing to the generalized toxicity of the substance Santavy and Reichstein (50) succeeded in isolating purified alkaloids from the mixture present in *Colchicum autumnale* Among these an al kaloid designated as Demecolcine (deacetylmethylcolchicine Colcemid[1]) proved of interest because of its lowered general toxicity (Fig 1)



Colchicine



Demecolcine

FIG 1

We started our trials of the new derivative first on animals and seeing a distinct inhibitory effect on granulocytopoiesis in cats began to use it in patients suffering from chronic myeloid leukemia in the autumn of 1952 Since that time many have employed the new drug and more than fifty nine papers concerning this compound have appeared in the literature

*Pharmacological Properties* Demecolcine is a crystalline water soluble alkaloid obtained from the seed of the *Colchicum autumnale* plant The normal acetyl group is replaced by a methyl group melting point 180 to 186

*Experimental Work* As we have demonstrated before (43) the cat is

[1] Ciba Co Basel Switzerland

terruption the maintenance dose is started which usually corresponds to one third of the dose needed before This maintenance dose is individually very different and lies between 3 and 4 mg for most cases But some cases being in a late phase of their disease need up to 6 8 and exceptionally even 10 mg daily In the beginning the leukocytes have to be counted daily When the maintenance dose has once been established leukocyte counts may be performed twice weekly and later once weekly or even twice monthly It is better to maintain the leuko cyte level between 12,000 and 15 000 and not to depress the leukocyte level to a completely normal count. This is also true for the other cyto static treatments We have five patients who have taken Colcemid con tinuously now for 3 years and who are still perfectly able to do their daily work. But some of the patients may develop resistance after a *more or less long time of treatment, as they also do for other drugs and* one may have to change to another cytostatic i e Myleran

*Tolerance and Side Effects* The tolerance to the drug in this dosage is usually excellent Patients show no loss of appetite on the contrary the appetite increases as the general condition improves In a dosage of 4 to 8 mg daily we have never encountered nausea or vomiting Some nausea and vomiting may occur in a daily dosage of more than *10 mg or after intravenous administration but we dont feel this is* normally necessary Bock and Gross (7) have given up to 20 mg orally daily but we feel that this is too toxic and may lead to disagreeable side effects In a dosage of 2 to 6 mg daily no alopecia occurred but going up to 8 and 10 mg one may observe alopecia in some cases and with more than over 10 mg it is usual for some alopecia to occur Neverthe less the hairs start growing again when the drug is reduced or suspended *for a certain time After the higher dose* azoospermia may occur as with Myleran or other cytostatics Unlike the normal colchicine Col cemid produces no gastric symptoms and no diarrhea This is confirmed by many other authors (1 6 8 9 18 20 21 23 24 28 30 32 33 35 36 46-48 53 57 58)

The thrombocyte and erythrocyte counts showed no depression in our cases with long term treatment in comparison to treatment with My *leran where some cases may show thrombocytopenia (Hansen 24b* and others) The hemoglobin and the erythrocyte count usually undergo a rise If the patient is already in a late phase showing myeloblastic transformation anemia may persist and necessitate transfusion

*Myeloblastic Transformation of Chronic Myeloid Leukemia* Most chronic myeloid leukemias show myeloblastic transformation at the end

**Clinical Tests**

CHRONIC MYELOGENOUS LEUKEMIA

Until now a total of thirty patients have been treated and some of them followed over a period of 3½ years A distinct depressive action was seen on the pathologically increased leukocyte levels together with a decrease in the size of the enlarged spleen and improvement of the general condition and hemoglobin level in twenty eight of thirty patients Comparison of the sternal marrow and the splenic puncture prior to therapy and after 3 months of treatment showed a marked change from immature myeloid to more mature forms This change is similar to that described in our investigations of arsenic compounds and urethan (39 41) and also occurs with the other cytostatics as for example Myleran During treatment many degenerative mitoses in the anaphase can be found in the bone marrow and spleen puncture (6)

Since our first report many other papers have appeared in the literature (1 6 8 9 18 20 21 23 24 28 30 32 33 35 36 46 48 53 57 58)

*Dosage* In our experience it is best to start with a dose of 4 mg of Colcemid daily divided into two doses given orally morning and evening There is great individual sensitivity of patients to this drug In some patients the leukocyte count comes down on a dose of 4 mg daily whereas others need a higher dose If the patient shows no effects after 10 days the dose may be increased to a daily intake of 6 mg A few patients need higher doses of up to 10 mg of Colcemid daily So if no distinct drop in the leukocytes occurs after 2 or 3 weeks of treatment with the smaller dosage the amount can be individually increased to 8 or 10 mg

Some patients with chronic myeloid leukemia who are in the late phase of their disease and already showing many blast forms need higher doses But in these cases too it is better to start with a smaller dose and to increase slowly If no effect occurs or if the patient is in a poor state and needs a very quick response one can use the i v injection of the preparation which acts much more rapidly One may start with 5 mg i v and count the leukocytes daily If necessary the i v dose can be increased to 10 mg daily (injection of the diluted drug —in dextrose solution—slowly in 5 to 10 minutes sometimes a slight nausea occurs)

*Maintenance Dose* As soon as the total leukocyte count has dropped to a level of 30 000 medication is stopped for 3 or 4 days The leukocyte level usually continues to drop for the next 5 to 6 days After this in

*Conclusion* Colcemid has now been on trial for 3½ years in our patients with chronic myeloid leukemia It has the advantage of being very well tolerated and the patient can take therapeutic doses for probably many years without showing any side effects *Especially it has to be stressed that in none of our patients observed until now as well as in those described in the literature has thrombocytopenia occurred* No depressive effect on erythropoiesis by the maintenance dose used has been observed. Colcemid may also have a great advantage in comparison to other cytostatic drugs in the treatment of chronic myeloid leukemia in that it will often still be active in the late myeloblastic transformation phase The disadvantage of Colcemid is that the drug shows a more individualized response than for example Myleran So in the beginning the leukocyte count must be checked daily and later on weekly and eventually every fortnight. But for long term treatment this disadvantage of more frequent blood examinations may be compensated for by the good tolerance of the drug and the excellent general condition of the patient. Alopecia may sometimes occur with higher dosages or when the patient is especially sensitive It is always a transient episode and the hair will grow again In the thirty patients treated this inconvenience was only seen twice in patients receiving over 10 mg daily

### Chronic Lymphatic Leukemia

On the basis of the animal experiments the main action of Demecolcine was expected to be on granulopoiesis and not on lymphopoiesis This was confirmed in our clinical trials in the treatment of lymphatic leukemia *The drug should therefore not be used in such cases*

In this group two of the most impressive clinical results were as follows

The first patient a 72 year old male had a diagnosis of typical lymphatic leukemia (old lymphocytic cell type) combined with lymphosarcoma exhibiting extremely pathological cells in the gland punctures There was a good response in the treatment of the lymphosarcoma attended by a slight decrease in the lymphocytes but he developed agranulocytosis of 10 days duration necessitating blood transfusions and antibiotics

The second patient was a 57 year-old male who was admitted to the hospital in an extremely poor general condition Blood values were 1 7 million erythrocytes with 39% hemoglobin leukocytes 190 000 with 96% lymphocytes Sternal puncture revealed infiltrated

of their disease Of five cases with this terminal myeloblastic transformation three have responded very well to Colcemid therapy Usually higher doses are necessary in these cases sometimes up to 10 mg and if no effect is seen orally one may change to the i v treatment for some time This observation that Colcemid may be active in the latest phase where Myleran usually no longer has an effect has been confirmed by other authors as in one case by Luhrs (36), another case by Pribilla and Stollberg (48) and in one case of Piney (47) Leonard and Wilkinson (32) had two cases with myeloblastic transformation which failed to respond We think the dosage used was too low however so no conclusions can be reached Bock and Gross (6) saw no result in these cases

Three cases with terminal blast transformation will be discussed The first patient came in in a very poor condition he had been resistant to x ray treatment and Myleran before He showed a marked improvement in general condition and also in the blood picture Nevertheless the hemoglobin and the erythrocyte count remained unchanged The second case came in with a severe bleeding of the gums and also in poor general condition He has now been working for a year with a daily intake of between 6 and 8 mg of Colcemid

The third was another type of myeloid leukemia with bleeding The patient came in with over 500 000 cells 10% blasts and a total of 54% very immature forms Only the very large spleen led us to suppose that he had suffered from a chronic myeloid leukemia before which had never been treated He had been very tired and in poor condition for the last 3 years This patient a boy of 16 years needed very high doses of Colcemid in relation to his body weight which was only 39 9 kg He required i v treatment in the beginning with a dosage of 10 mg daily which was reduced and then stopped for a short time The blast forms returned very quickly *He has now been maintained on 8 to 10 mg daily for 1½ years and he is still in very good health and going to school* This patient lost his hair twice when the high dosage was used i v Two other patients with an acute myeloblastic transformation who did well under previous treatment with maintenance doses of Colcemid failed in a later phase to respond to higher doses of Colcemid and also did not respond to Myleran They showed a slight improvement with 6 mercaptopurine (Purinethol) *Personally I think that the great advantage of Colcemid is the fact that some of the myeloblastic transformations which often occur in the later stages of chronic myeloid leukemia may still respond to Colcemid treatment but usually not to other cytostatic drugs*

*Conclusion* Colcemid has now been on trial for 3½ years in our patients with chronic myeloid leukemia It has the advantage of being very well tolerated and the patient can take therapeutic doses for probably many years without showing any side effects *Especially it has to be stressed that in none of our patients observed until now as well as in those described in the literature has thrombocytopenia occurred* No depressive effect on erythropoiesis by the maintenance dose used has been observed Colcemid may also have a great advantage in comparison to other cytostatic drugs in the treatment of chronic myeloid leukemia in that it will often still be active in the late myeloblastic transformation phase The disadvantage of Colcemid is that the drug shows a more individualized response than for example Myleran So in the beginning the leukocyte count must be checked daily and later on weekly and eventually every fortnight But for long term treatment this disadvantage of more frequent blood examinations may be compensated for by the good tolerance of the drug and the excellent general condition of the patient *Alopecia may sometimes occur with higher dosages or when the patient is especially sensitive* It is always a transient episode and the hair will grow again *In the thirty patients treated this inconvenience was only seen twice* in patients receiving over 10 mg daily

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marrow After receiving only 65 mg over a period of 13 days he died in an acute lymphatic exacerbation with a terminal cell rise to 400 000 He also developed a severe necrotic tonsilitis

*Our experience with Colcemid in lymphatic leukemia shows that its use here is strictly contraindicated* Other investigators i e Leonard and Wilkinson (32) and Bousser and Christol (9) have had the same experience and have also seen some very severe exacerbations or granulocytopenic reactions Only Storti and Gallinelli (55) believe that Colcemid may be useful in some cases in a low dosage and with frequent counts of the granulocytes The severe exacerbation which was found in the second patient with lymphatic leukemia discussed above and the similar observations by Leonard and Wilkinson (32) lead to the conclusion that the drug should not be used in chronic lymphatic leukemia

## Acute Blast Cell Leukemia

The clear therapeutic success in chronic myeloid leukemia especially in some of the blast cell transformations in the late phase of the disease made us consider using the drug also in primary acute subleukemic leukemia One case of a 33 year old male is showing a definitely good response The essential findings of this case will be described

*Physical Findings* The general condition was poor Spleen and lymph glands were not palpable Liver was slightly enlarged to below the costal margin RBC 856 000 with 25% hemoglobin color index 1 47 There were 5000 leukocytes with 16% blasts thrombocytes 17 100 Sternal marrow showed 50% blast forms with large nuclei and relatively little cytoplasm

*Clinical Course* After daily treatment with 5 mg of Demecolcine and blood transfusions the patient showed a distinct clinical remission Although the blast forms continued to constitute about 50% of the cells in the sternal marrows there were only occasional blast cells in the peripheral smears

Resistance to Demecolcine developed in the fifth month manifested by a new rise in the leukocytes and blast forms with the simultaneous appearance of thrombocytopenic purpura 6 Mercaptopurine (Purinethol) was instituted resulting in a new remission The patient died 12 months after the onset of his disease

Other cases however have not shown the same result although one case (an adult male) which I saw personally in the department of Professor Waldenstrom in Malmo Sweden also showed a very good response but developed resistance after 12 months Demecolcine may be tried in some cases which fail to respond to other drugs Leonardi and D Angolo (33) treated seven cases of acute blastic leukemia with no result Storti and Gallinelli (55) and Bock and Gross (6) also obtained no results *The main field of Demecolcine is restricted to chronic*

*myeloid leukemia and the terminal blast cell transformation of chronic myeloid leukemia but not to the primary acute blast cell leukemia*

**Other Diseases**

Twenty two cases of carcinoma and sarcoma were treated The daily dosage varied from 3 to 7 mg Side effects with granulocytopenia occurred in seven cases with a drop of the granulocytes in one case to 499 (a total of 870 leukocytes) No other toxic effects were observed Although nausea and emesis were present in six cases prior to therapy none could be attributed solely to the Colcemid A definite therapeutic effect was not observed in any of these cases

In a case of hypernephroma with metastases in the lungs and bones the temperature which was increased before fell to normal and an obvious improvement of appetite occurred but the tumors in the lungs remained unchanged Four cases of lymphosarcoma showed a slight regression of the lymph nodes but no definite remission as was the case with TEM which was employed later Four myeloma patients showed no effect In the literature Volterra and Romualdi (57) have mentioned one case of parotid tumor and one of ovarian carcinoma which seemed to respond to Demecolcine treatment

*Hodgkin's Disease* Although we have used the drug in only three cases we do not have the impression of having produced any definite effect on the disease Storti and Gallinelli (55) however have reported good results in a long term treatment with small doses of 2 to 4 mg daily This has been confirmed by Gigante (23) Volterra and Romualdi (57) and Zbinden (59) All these authors recommend Colcemid to be used when other treatments have been abandoned Zbinden (59) has reported an interesting case of Hodgkin's disease with lung metastases which responded very well to combined treatment of Colcemid and x ray He injected the Colcemid (4 to 6 mg) i v 6 to 8 hours before the x ray treatment He thinks that the action of Colcemid in arresting mitoses in the anaphase tends to make the patient more sensitive to the later x ray treatment

*Skin Tumors* Good results with an application of Colcemid ointment to the skin in neoplastic transformation have been reported by several authors The results were especially striking in Bowen's precancerosis and also sometimes impressive in basal cell carcinoma Agostini (2) Cottini and Randazzo (16) Berres (5) and Midana and Ormea (38) saw good results from injecting the Colcemid directly into the tumors of mycosis fungoides No results were seen in lupus erythematosus by Volterra and Romualdi (57)

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## REFERENCES

1 Abou Chaar C Lebanese Pharmaceut J 2 117 (1954)
2 Agostini A Arch ital dermatol sifilog e venereol 27 271 (1955)
3 Antoine B Vie med 36 995 999 (1955)
4 Baer B and Arndt, A Klin Wochschr 32 109 (1954)
5 Berres H H Arch Dermatol u Syphilis 197 479 (1954)
6 Bock H E and Gross R Acta Haematol 11 260 (1954)
7 Bock H E and Gross R Klin Wochschr 31 816 (1953)
8 Bock H E and Gross R Arztl Wochschr 9 344 (1954)
9 Bousser J and Christol D Presse med 63 1109 (1955)
10 Bucher O Z Zellforsch u mikroscop Anat 29 283 (1939)
11 Cattaneo I Vercillo L and Mannino-Sigillo A Arch "E Maragliano patol e clin 10 681 (1955)
12 Cattaneo I Vercillo L and Mannino-Sigillo A Arch E Maragliano patol e clin 10 901 (1955)
13 Cattaneo I Vercillo L and Mannino-Sigillo A Arch E Maragliano patol e clin 10 915 (1955)
14 Cattaneo I Vercillo L and Mannino-Sigillo A Arch sci med 80 99 (1955)
15 Colsky J Wallace St and Barowitch M M New Engl J Med 253 730 (1955)
16 Cottini G B and Randazzo S D Dermatologica 110 426 (1955)
17 Cottini G B and Randazzo S D Dermatologia (Napoli) 6 167 (1955)
18 Ducla Soares A and Paludo Valente M Gaz med portuguesa 8 281 (1955)
19 Dustin A Arch exptl Zellforsch Gewebezucht 22 395 (1938)
20 Fleischhacker H Munch med Wochsch 97 191 (1955)
21 Fromowicz, K K Polski Tygodn k Lekarski 10 602 (1955)
22 Gardini G F and Rizzente S Tumori 40 625 (1954)
23 Gigante D Boll soc ital emat 1 2 109 (1954)
24a Gigante D Le Sang 26 404 (1955)
24b Hansen P B in Leukaemia Research" p 205 J & A Churchill London 1954
25 Homann W and Otto H Z Krebsforsch 60 730 (1955)
26 Jacob W and Gopfert H Munch med Wochschr 97 1175 (1955)
27 Jacoby C Arch exptl Pathol Pharmakol 27 129 (1890)
28 Kaiser E Nouv med (Belg) 4 67 (1955)
29 Keck K and Kaiser E Oesterr botan Z 101 579 (1954)
30 Keibl E Wien klin Wochschr 67 511 (1955)
31 Landolt R F Deut Arch klin Med 191 378 (1943-44)
32 Leonard B J and Wilkinson, J F Brit Med J 4918 874 (1955)
33 Leonardi P and D Angelo B Acta Med Patavina 14 69 (1954)
34 Lachtman A Dissert tion University of Zurich (1954)
35 Loffler W and Bollag W Oncologia 8 70 (1955)
36 Luhrs W Deut Gesundheitsw 10 1365 (1955)
37 Meier R Schar B and Neipp L Experientia 10 74 (1954)
38 Midana A and Ormea F Minerva dermat 1 29 1 (1954)
39 Moeschlin S Helv Med Acta 15 107 (1948)

*Gout* Very good results can be obtained in the acute phase of gouty arthritis We have seen prompt relief in 6 to 12 hours in three cases with a single dose of 8 mg orally And Colsky *et al* (15) obtained good remissions starting with 1 mg every hour up to a total of 8 mg We prefer the total dose of 8 to 10 mg given in a single dose at the start of the attack

## Summary

Colcemid an alkaloid isolated by Santavy and Reichstein (50) in 1950 from *Colchicum autumnale* shows a toxicity one thirtieth that of colchicine in animal experiments and a marked selective depressive effect on granulocytopoiesis *Clinical trials which have now been in progress for 3½ years and the confirmation of many other authors show that Demecolcine can be recommended for a long term oral therapy of chronic myeloid leukemia* The good tolerance and the lack of toxic side effects with the dosage recommended may be stressed The initial dose should be 4 mg daily and should slowly be increased to 6 to 8 mg daily and in some severe cases even up to 10 mg daily The drug should be stopped for 3 or 4 days after the leukocytes have dropped to 30 000 and then may be resumed at a maintenance level which varies between 3 and 4 mg in most cases In some cases however the maintenance dose has to be increased to 6 mg and in a few cases even to 10 mg daily *The great advantage of the drug is that it may also be useful in some late cases with terminal blast cell transformation*

No side effects on other blood cells have so far been observed Especially it should be stressed that no thrombocytopenic effect has been seen in all the cases observed or reported in the literature up to now *Alopecia may occasionally occur if a high dosage is used or if the patient* is especially sensitive But normal hair growth has returned in all cases As in other cytostatics (nitrogen mustard Myleran arsenic etc ) there is an inhibition of spermatogenesis and probably ovulation (one case at autopsy and our animal experiments) The side effects are unimportant in comparison to the good clinical effect on the leukemia however

*Colcemid is strictly contraindicated in lymphatic leukemia* producing sometimes a very striking exacerbation of the disease or severe granulocytopenia which may even be fatal Unfortunately Colcemid administered orally or intravenously has not proved a definite therapeutic success in the therapy of other neoplasms (myeloma lymphosarcoma Hodgkin s carcinoma and sarcoma) and cannot be employed in a sufficiently high dose because of its leukopenic effect It may however be helpful for tumor injection and external application in the case of some skin tumors

cases included. There are shown Dr. Wintrobe's results through 1954. There is a more recent publication which increases his experience but the results are, he tells me, comparable to those reported in 1954. As can be seen, there is excellent general agreement in the results obtained by the several investigators. Between 80 and 90% of individuals with chronic myeloid leukemia are benefited by the drug and the duration of remission is from zero time in those individuals who do not respond up to 48 months.

In our experience we have used this agent only when the patient is symptomatic, as has been indicated by Dr. Moeschlin and Dr. Dameshek. I am not so sure as they are that this is necessarily right. I don't believe we have the evidence.

TABLE I
TREATMENT OF CHRONIC MYELOID LEUKEMIA WITH MYLERAN

| Investigator | Number of cases | Daily dose mg | Remissions Number | Remissions % | Duration of remission months |
|---|---|---|---|---|---|
| Galton (1955) | 30 | 4–10 | 26 | 86 | 0–48 |
| Shimkin (1954) | 11 | 2–25 | 7 | 64 | 0–19 |
| Wintrobe (1955) | 16 | 4–6 | 14 | 88 | 2–21 |
| Gigante (1955) | 14 | 10–20 | 12 | 86 | 0–5 |
| Pribilla (1955) | 10 | 2–6 | 9 | 90 | 4–15 |
| Ramioul (1955) | 5 | 2–8 | 3 | 60 | – |
| Suarez (1955) | 3 | 4–8 | 3 | 100 | 4–9 |
| Videbaek (1955) | 7 | 6–12 | 6 | 86 | 2–16 |
| | 96 | 2–25 | 80 | 83 | 0–48 |
| Delafield Presbyterian group (1955) | 21 | 2–20 | 17 | 81 | 0–48 |
| Total | 117 | 2–25 | 97 | 83 | 0–48 |

It is possible, with an agent as easily administered as Myleran, that it would be worth while to accumulate evidence to find out whether therapy of an individual as soon as the diagnosis is made may improve the over-all results. Perhaps this would take too many cases and too much time. I don't think we are permitted a statement such as has been made, however, that one must withhold therapy until the patient is symptomatic.

DR. DAMESHEK: We don't do that except in the very benign mild cases of lymphocytic leukemia. In the chronic granulocytic cases we always start treatment immediately.

40 Moeschlin S *Acta Haematol* **1** 225 (1948)
41 Moeschlin S "Spleen Puncture Grune and Stratton New York 1951
42 Moeschlin S *Praxis* **43** 66 (1954)
43 Moeschlin S and Bodmer A *Blood* **6** 242 (1951)
44 Moeschlin S Meyer H and Lichtman A *Schweiz med Wochschr* **83** 990 (1953)
45 Moeschlin S Meyer H and Lichtman A in Leukaemia Research p 216 J & A Churchill London 1954
46 Motz G *Ars Med Wien* **45** 105 (1955)
47 Piney A *Acta Haematol* **14** 83 (1955)
48 Pribilla W and Stollberg G *Deut med Wochschr* **80** 1027 (1955)
49 Ribuffo A *Dermatologia* **6** 6 (1955)
50 Santavy F and Reichstein T *Helv Chim Acta* **33** 1606 (1950)
51 Scaffi M and Baldassarri R *Ga*z *intern med e chir* Anno 59 **60** Nos 9 10 (1955)
52 Schar B Loustalot R and Gross F *Klin Wochschr* **32** 49 (1954)
53 Schulten H and Pribilla W *Med Klin* (*Munich*) **50** 1631 (1955)
54 Steinmann F *Rev suisse zool* **61** 163 (1954)
55 Storti E and Gallinelli R *Schweiz med Wochschr* **84** 612 (1954)
56 Uffer A Schindler O Santavy F and Reichstein T *Helv Chim Acta* **37** 18 (1954)
57 Volterra M and Romualdi G *Boll Soc Ital Ematol* **2** 197 (1954)
58 Volterra M Romualdi G and Abbozzo G *Giorn Clin Med* (*Parma*) **36** 627 (1955)
59 Zbinden F *Schweiz med Wochschr* **85** 994 (1955)

CHAIRMAN BETHELL I should like to ask Dr Gellhorn and Dr Doan who will speak next on the subject of chronic leukemias with special reference to chemotherapy at the time they report their results to attempt to define the usefulness of the drugs under discussion in relation to other agents which have been available for a long time

In particular we should like to know how Myleran in the treatment of chronic granulocytic leukemia and Chlorambucil or CB 1348 in the management of chronic lymphocytic leukemia and Hodgkins disease compare with ionizing radiation and what indications there may be in the speakers experience for the choice of a drug instead of radiation and vice versa

Dr Gellhorn will you begin?

DR GELLHORN Since Dr Dameshek was good enough to leave something for me to say and since he didnt bring a lantern slide I should like to summarize his results with Myleran

The results of a number of investigators are summarized in Table I which is a bit out of date and does not have Dr Dameshek s thirty four

cases included There are shown Dr Wintrobe's results through 1954 There is a more recent publication which increases his experience but the results are he tells me comparable to those reported in 1954 As can be seen there is excellent general agreement in the results obtained by the several investigators Between 80 and 90% of individuals with chronic myeloid leukemia are benefited by the drug and the duration *of remission is from zero time in those individuals who do not respond* up to 48 months

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DR GELLHORN Thank you Dr Dameshek

At our clinic (1) Myleran is given in doses of 10 mg daily and treatment is continued usually until the count has fallen to approximately 25% of its initial level Thus if the leukocyte count starts at 400 000 we pause at the time the count has fallen to 100 000 to see whether it will fall further We are also guided by the slope of the fall If the fall is very precipitous then therapy may be withheld to find out the level to which leukocytes will decrease

Like Dr Dameshek we feel it is important to take the count to a normal level before discontinuing therapy Unlike Dr Dameshek we do not use maintenance therapy except in those individuals who have demonstrated that there will be a prompt rise in the count Some individuals fall into this group and others remain in remission for periods measurable in terms of a year or more

We have administered the compound to patients with other malignant diseases including various types of epithelial tumors but without any effect I hope that in the discussion Dr Bierman will comment on the use of this agent in the treatment of acute leukemia or the blastic crisis of chronic myeloid leukemia

The other compound about which there has been comment this evening is CB 1348 which is now being distributed in this country for investigational use by Burroughs Wellcome under the name of Chlorambucil It is a nitrogen mustard related to the compound HN2 and is related as well to triethylene thiophosphoramide also called thio TEPA This agent is active on oral administration It has been studied in a variety of lymphomas

Our experience has been primarily in the treatment of chronic lymphatic leukemia and to date we have studied eighteen patients (2) The results do not approach those of Myleran in terms of efficacy We had three individuals who fell into the category of excellent objective remission which means no evidence of disease There are eight individuals in whom the improvement was considered good in these patients the differential count did not return to normal There were nine individuals who had essentially no improvement

Interestingly enough in spite of the fact that there were eleven good responses in these eighteen patients the subjective improvement was not so satisfactory as the objective In other words we were treating in some cases the peripheral count rather than the patient

I am unable to answer the question posed by our Chairman namely

the advantage of this compound over radiotherapy because there is no quantitative evidence on that to the best of my knowledge

One of the chief advantages of Chlorambucil over triethylenemelamine is that it produces no unpleasant subjective side reactions in the patient There is no nausea or vomiting Triethylenemelamine you know does have such side reactions Further we have found that this is a relatively less toxic compound in terms of depressing platelets to hazardous levels We have not observed this in any one of the patients thus far treated and therefore we feel that it is a somewhat safer agent to use than triethylenemelamine for patients on an ambulatory basis

We have not observed any significant complications with this agent other than in one individual who had a rapid fall of the peripheral count He was painting his barn in the hot summer and developed hyperuricemia with complete renal shutdown which amazingly was reversed by nephrostomies and washing out of large amounts of urate crystals from the pelvis This is the primary complication that we have observed with Chlorambucil

REFERENCES

1 Hyman G A and Gellhorn A *J Am Chem Soc* in press
2 Ultmann J E Hyman G A and Gellhorn A *J Am Chem Soc* in press

Chairman Bethell I think it is a complication that may be observed with any agent capable of producing intense lysis of leukemic cells

Dr Doan would you like to continue?

Dr Doan It has been established that nitrogen mustard and to a lesser extent TEM are recognized supplements to radiotherapy in the treatment of disseminated Hodgkin s disease and in some stages of other related lymphomas and leukemias Nitrogen mustard has the disadvantages however that it must be administered intravenously that it produces marked and at time irreversible bone marrow depression and that it may cause such side effects as malaise nausea vomiting and enteritis Triethylenemelamine (TEM) which may be administered by mouth is often very toxic and its effects unpredictable

During the past eight years a large series of aromatic derivatives of the nitrogen mustards have been synthesized and screened at the Chester Beatty Research Institute Royal Cancer Hospital London (3) The most promising one of these compounds thus far has been designated

CB 1348 it is p bis (2 chloroethyl)aminophenylbutyric acid a limited supply of which compound has been provided to Dr B K Wiseman Dr B A Bouroncle and myself through the courtesy of Professor Alexander Haddow Director of the Institute

A total of fifty five selected patients have been treated in our leukemia and lymphoma clinics over the past 15 months There were thirty patients with tissue verified Hodgkin s disease thirteen with monocytic leukemia two with reticulum cell sarcoma one with lymphosarcoma three with chronic lymphatic leukemia one with acute lymphatic leukemia one with multiple myeloma three with metastatic carcinoma and one with mycosis fungoides There were twenty nine males and twenty six females and their ages ranged from 2½ to 77 years

The compound was administered by mouth as 2 mg tablets given preferably before meals two or three times daily

The initial dose of CB 1348 in most patients was 0 3 mg /kg /day for a total period of 21 days In most patients treatment was then stopped A second course of therapy was administered when symptoms of activity recurred The dosage was either the same as the initial dosage or more frequently only 0 2 mg /kg /day for 21 days All patients after the second course and most patients after the initial course were kept on a maintenance dosage of CB 1348 of 0 05 mg /kg /day

A few patients complained of nausea and anorexia during the course of therapy Only in one case however did these complaints necessitate discontinuation of the treatment Several patients complained of "nervousness" during therapy This symptom disappeared when CB 1348 was discontinued or when the patients were changed to a maintenance dose

Most patients developed a transitory slight to moderate leukopenia anemia and thrombocytopenia The maximum depression was usually observed 6 weeks after treatment was initiated In therapeutically effective dosages however the depressant effect on the bone marrow was surprisingly moderate and rapidly reversible even in patients who already showed a hypoplastic marrow as a result of previous treatments

Our results in patients with Hodgkin s disease on the whole have been encouraging Thirty patients (seventeen males and thirteen females) with an age range from 12 to 64 years have been treated with CB 1348 The diagnosis in all cases was confirmed by lymph node biopsy The duration of illness before CB 1348 therapy was initiated was from 3 months to 14 years With the exception of one patient all had received some form of treatment previously twenty eight had received from one

to seven courses of deep x ray therapy in the evolution of their disease fifteen patients had had from one to seven courses of nitrogen mustard and six had been given TEM Three had undergone surgery for complications related to Hodgkin s disease two of them had required splenectomy and one had a laminectomy with removal of an epidural Hodgkin s mass

All thirty patients had developed signs and symptoms of generalized disease All but five were in far advanced stages of the disease Most of the patients had previously failed to respond to x ray nitrogen mustard and TEM therapy or were not suitable for nitrogen mustard or TEM therapy because of marked depression of the hematopoietic system The results of treatment in these patients with Hodgkin s disease are summarized in Table I One patient is listed as having drug intolerance Administration of the drug was discontinued because of marked nausea One other patient in this group complained of this symptom but only to a mild degree

Seven of the patients failed to respond to the first course of CB 1348 therapy Two of these patients were terminal" cases who received only eight days of therapy One had parenchymal infiltration of the lungs which progressed during therapy and the other two showed progression of neurologic signs and symptoms despite CB 1348 therapy

Four patients showed "some effect" due to medication in the sense that their general condition and some of the symptoms improved but this improvement lasted for less than 2 months Eleven patients are listed as "improved" By this is meant that the drug induced a remission of real value lasting from 2 to 4 months Five of these patients received a second course of CB 1348 one had a second complete remission which lasted 7 months and is at present receiving the third course of this medication and one had a second remission which has been in progress for 1 month The other three failed to respond to the second course of therapy

Seven patients with Hodgkin s disease are listed as having a "good" remission By this is meant that the drug induced an improvement of real value to the patient so that as a result of therapy something approaching a return to normal life was achieved for a period of 4 months or longer Three of these patients are still in their first remission for 4 to 7 months The other four patients obtained a remission after a second course of CB 1348 One of them is in a second remission of 10 months duration at present The second patient received a third course and is in a third remission at present for 3 months One other patient

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failed to respond any longer to CB 1348 or to x ray therapy and died on December 30 1955 Galton *et al* (2) have continued to obtain success ful remissions after as many as six courses of CB 1348 therapy

The favorable effects in patients who responded to CB 1348 included a rapid return of the temperature to normal usually during the third to twelfth day of therapy lymphadenopathy splenomegaly and hepa tomegaly decreased during therapy with maximum improvement ob served approximately 6 weeks after the treatment was initiated a progressive gain in weight was observed in all patients who thus re sponded to therapy jaundice hepatomegaly and liver functions were improved in two patients who presented such findings before therapy was initiated

The following is a summary of the case history and graphic representa tion of the course of the illness in one selected patient who had an ex cellent remission from CB 1348 treatment

*Case No 2* M K a 43 year-old white woman was diagnosed as having Hodgkin s disease by biopsy of a cervical lymph node in 1953 She received treatment with nitrogen mustard three courses of TEM three courses of deep x ray therapy over abdominal right cervical and supraclavicular lymph nodes and spleen and cortisone and butazolidine The patient gradually became refractory to all previous therapy In January 1954 she presented with fever anorexia marked generalized lymphadenopathy and marked splenomegaly Her weight was 119 pounds Her peripheral blood study showed 1 590 000 red blood cells per cubic millimeter 3800 white blood cells per cubic millimeter blood platelets 17 660 per cubic millimeter and 6 3 g of hemoglobin per 100 cc The patient was transfused before therapy was started She was given CB 1348 20 mg daily (0 4 mg kg ) for 30 days Her fever abated after 3 days of this therapy The enlarged lymph nodes and spleen regressed She gained 41 pounds The red blood cell level rose and remained about 3 5 million per cubic millimeter hemoglobin rose to 11 8 g % The remission lasted for 5 months At the end of this time she relapsed with 100 F temperature slight lymphadenopathy and recurrent splenomegaly She was then given CB 1348 6 mg daily for 21 days followed by a continuing daily maintenance dosage of 3 to 4 mg Her second complete remission has now been in progress for 10 months (Fig 1)

Thirteen patients—six males and seven females age range from 18 to 77 years—with blood and bone marrow diagnoses of acute monocytic leukemia were treated with CB 1348 All had marked anemia and

TABLE I

RESULTS OF TREATMENT WITH CB 1348 IN FIFTY FIVE PATIENTS

| Diagnosis | Number of patients | Results: First remission: Good | Im proved | Some effect | Failure | Drug intol | Second remission: Number of patients | Good | Im proved | Failure | Third remission: Number of patients | Im proved | Failure |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hodgkin's disease | 30 | 7 | 11 | 4 | 7 | 1 | 9 | 5 | 1 | 3 | 2 | 1 | 1 |
| Monocytic leukemia | 13 | 1 | | 4 | 8 | | | | | | | | |
| Chronic lymphatic leukemia | 3 | | | 1 | 2 | | | | | | | | |
| Acute lymphatic leukemia | 1 | | | | 1 | | | | | | | | |
| Reticulum cell sarcoma | 2 | 1 | | 1 | | | | | | | | | |
| Lympho sarcoma | 1 | | | | 1 | | | | | | | | |
| Mycosis fungoides | 1 | | | | 1 | | | | | | | | |
| Multiple myeloma | 1 | | | | 1 | | | | | | | | |
| Metastatic carcinoma | 3 | | | | 3 | | | | | | | | |
| Total | 55 | 9 | 11 | 10 | 24 | 1 | 9 | 5 | 1 | 3 | 2 | 1 | 1 |

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Fig 1 Hodgkin's disease of three years duration refractory to conventional therapy and in advanced stage of the disease showing two good remissions on CB 1348 Second remission in progress 10 months

thrombocytopenia In all cases the bone marrow showed marked replacement *(60 to 90%) of normal elements by monoblasts One patient* had received HN2 Two had been previously treated unsuccessfully with TEM The other ten patients had received no therapy before CB 1348 was started

Eight patients failed to respond to CB 1348 medication Each of five patients of this group then subsequently received a course of HN2 without improvement Four patients listed as showing "some effect" exhibited a temporary decrease of white blood cells but without improvement in red blood cells platelets or percentage of monoblasts in the bone marrow After a course of CB 1348 22 mg daily (0 3 mg /kg ) for 14 days a complete remission still in progress for 10 months was obtained in one patient This patient was a 45 year-old white female who had complained of weakness for 5 months Laboratory findings included *red blood cells 2 51 million per cubic millimeter white blood* cells 1250 per cubic millimeter hemoglobin 9 1 g % platelets 90 400 per cubic millimeter reticulocytes 5 2% The bone marrow samples showed almost complete replacement of normal elements by monoblasts After therapy she required no further transfusions The peripheral blood now contains red blood cells 4 17 million per cubic millimeter white blood cells 5950 per cubic millimeter with a normal differential hemoglobin 14 4 g per 100 cc platelets 800 640 per cubic millimeter reticulocytes 3 4% *The bone marrow has become essentially normal*

Two patients with reticulum cell sarcoma were treated A 64 year old white male with reticulum cell sarcoma diagnosed by an abdominal lymph node biopsy was treated with CB 1348 He showed progressive disseminated disease with profound weight loss At physical examination several large abdominal masses were palpable and large retroperitoneal and retrogastric tumors were demonstrated by x ray He received a course of CB 1348 16 mg (0 3 mg /kg ) daily for 21 days followed by *a maintenance dosage of 4 to 8 mg daily* The patient regained 27 pounds in weight and is now back at his normal activities A marked regression of the abdominal tumor masses was obtained His remission has been in progress for 7 months The second patient a 63 year old white female with a similarly diagnosed large abdominal tumor mass refractory to deep x ray treatment has had some clinical improvement with regression in the tumor on CB 1348 therapy for 12 weeks

Three patients with chronic lymphatic leukemia in advanced stages *of the disease characterized* by practically complete replacement of the normal elements of the bone marrow by lymphocytes with marked

neutropenia anemia and thrombocytopenia were treated They had become refractory to radioactive phosphorus and x ray therapy directed over the packed cell flat bones No further clinical or hematologic improvement was observed

One patient with acute lymphatic leukemia one with mycosis fungoides and one with multiple myeloma were treated unsuccessfully with CB 1348 It is concluded from the reported observations that CB 1348 is of some value in the treatment of selected patients with disseminated Hodgkin s disease as a supplement to x ray therapy and in an occasional case of monocytic leukemia and reticulum cell sarcoma

It has been a safer drug in our hands than triethylenemelamine (TEM) It is preferred over nitrogen mustard in some cases because it has practically no side effects and it has proved to be less damaging to the hematopoietic system

REFERENCES

1 Bouroncle B A Doan C A Wiseman B K and Frajola W J *Arch Internal Med* in press
2 Galton D A C Israels L S Nabarro J D N and Morwenna T *Brit Med J* 2 1172 (1955)
3 Haddow A *in* Leukaemia Research Ciba Foundation Symposium p 196 Little Brown and Company Boston 1954

CHAIRMAN BETHELL Thank you Dr Doan It is late but we should like to give an opportunity to the audience to ask any question We have now heard a report based on personal experience by each member of the panel and I shall ask for questions and invite discussion from the audience Dr Wintrobe perhaps you would care to comment now?

DR WINTROBE There is one comment I should like to make The impression seems to have been left that Myleran is without any toxic effects whatever I think it ought to be pointed out that Myleran is not without toxic effects that there may be thrombocytopenia associated with its use that even marrow aplasia perhaps can occur So like all the agents we are talking about there is good with the bad although I quite agree with the impression that has been given namely that it is an extremely useful agent for the treatment of chronic myelocytic leukemia

I should like to echo Dr Gellhorn s question also and should like to hear from Dr Bierman and would ask him to state his evidence concerning the effectiveness of Myleran in acute leukemia

CHAIRMAN BETHELL I have Dr Bierman in mind but are there any other members of the panel who would like to comment at this time?

DR. DAMESHEK I should like to end on a note of optimism in that not infrequently we do get some remarkable results in some of our cases of chronic lymphocytic leukemia—patients who start out with a very high white cell count and with many symptoms With the use of TEM there are often striking clinical and hematologic remissions which may last for several years In chronic granulocytic leukemia the consistency of the results with Myleran is indeed remarkable

Also even in *acute* leukemia where treatment is so often of pathetic benefit I occasionally find myself having feelings of optimism not only because of the excellent remissions so frequently obtained with aminopterin in the childhood type but because of the evidence occasionally found that the therapy has actually "burnt out" the leukemic process We autopsied three such cases Death was due to hemorrhage but there was no histologic evidence of the previous leukemic process Naturally these deaths are by no means what one can call good results However I must mention another child who was in complete remission for 4 years clinically and hematologically At the end of that time he developed a remarkable urogenital abnormality—stony enlargement of the testicles great thickening of the ureters and bladder—and eventually died of renal disease secondary to ureteral obstruction Owing to the continuous use of aminopterin the leukemic process had apparently been "burned out" leaving rests of leukemic cells in the kidneys ureters bladder and testicles Thus one may visualize in the I hope not too distant future the development of even more potent and specific chemicals that may conceivably burn out" a leukemic process and leave normal tissues intact, thus allowing long continued remissions At present, I think we must concede that the outlook for patients with acute leukemia is discouraging

DR SIDNEY FARBER Mr Chairman may I say something that I think ought to be said? Dr Dameshek has confused me If we today can have 10% of 560 children alive 30 months after the onset of acute leukemia and in so many instances in excellent health going to school and playing normally then that represents an achievement of major importance on the part of total care including the use of antileukemic agents

If we can have 50% alive at 14 or 15 months that represents another major achievement for those 50% of children for their parents for the doctors who take care of them and for the stimulation of research workers who hope to produce something better while these children are still

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I should like to echo Dr Gellhorn's question also and should like to hear from Dr Bierman and would ask him to state his evidence concerning the effectiveness of Myleran in acute leukemia

CHAIRMAN BETHELL I am certain that no one of us is satisfied with our present position in the treatment of acute leukemia. Nevertheless I feel that there is room for optimism for a hopeful progressivism

I am reminded of the fact that there was an outstanding symposium on the subject of hematology in 1939 at the University of Wisconsin I am quite sure that most of what we have said tonight is completely beyond any anticipation or opinions that prevailed at that time That was some 16 or 17 years ago I am equally confident that in less time than 16 years there will be held another symposium such as this in which even greater progress will be evidenced So I believe we can take heart but we certainly recognize the shortcomings of our present knowledge and therapeutic materials

Now Dr Bierman may we hear from you?

DR. HOWARD R BIERMAN (Duarte California) Apparently Dr Dameshek got me into this too asking me to give our experience with the treatment of acute granulocytic leukemia in a 40-year-old male

We have had the privilege of working with Myleran since the fall of 1951 when Dr Haddow sent us some of the original compound shortly after he Dr Timmis and Dr Galton had started to use it prior to their reports in England We now have in our combined experience approximately fifty cases the longest one of which is a man who has gone since October 1951 with apparently a very benign course

The original report by Dr Petrakis in 1953 recorded two cases of acute leukemia or subacute granulocytic leukemia with predominantly immature blasts in marrow and blood with survival for 6 months after therapy The experience since that time has been with eight comparable cases of either acute or subacute leukemia some with rather fulminating courses and I believe our experience now is about the same an average survival of about 6 months after initiation of therapy

I think what Dr Farber said earlier in connection with the acute leukemias should be given great consideration in chronic leukemias—that one should use all the available adjuncts to therapy including anti hemorrhagic agents and antibiotics—in an effort to support patients

Our experience with cortisone in cases of granulocytic leukemia has not been as happy as in lymphocytic leukemia We have seen six unfortunate situations which we think were exacerbations in chronic granulocytic leukemia using the corticoids either cortisone or hydrocortisone

As for the toxic effects of Myleran given over long periods of time in some instances for 2 or 3 years we have observed three cases of

alive I believe Dr Burchenal and all the rest of us on the panel who have worked with children in large numbers have had an experience that is not in accord with Dr Dameshek s

Dr William Dameshek There can be no question that the introduction of the folic acid antagonists for the treatment of acute leukemia is a major achievement One cannot quibble about that I think Dr Farber you should be congratulated on it However I do think that these agents are by no means the best of all possible therapeutic principles particularly in the adult group They represent simply the best therapy we have as of now If they *were* the best possible agents I think we would be getting far better results than the 50% survival at the end of 10 months or the 10% survival at the end of a year or two

Dr Sidney Farber May I make myself clear? I was talking about the folic acid antagonists the purine antagonists ACTH cortisone the compounds Dr Hill talked about and so on in addition to everything else we do for these children I was not talking about any one group These figures refer only to children and not to adults

Dr William Dameshek To continue I think it is truly wonderful that one can reverse the course of some cases of acute leukemia This is without doubt a major achievement and one which frankly I for one never expected to see when it was first reported The trouble with it is that every single one of these children or adults once they have obtained a remission will relapse whether in 2 months or in 2 years With very rare exceptions relapse is certain and they all go on to die Their last few months on earth are often a nightmare of porcine obesity painful legs bleeding and suffering parents

That is why I say it is of *pathetic benefit"* *benefit* because there is a temporary beneficial effect *pathetic* because there is simply a prolongation of the inevitable Also pathetic because it must be realized by now that both the folic acid antagonists and 6-mercaptopurine either alone or in combination with the steroids can do only so much and no more Better agents are undoubtedly around the corner and that is why I think we will eventually be able to keep leukemia under control When one realizes that leukemia can be reversed even temporarily I feel rather optimistic but when I see our present day results however good they may look on paper I am depressed This ambivalence of feeling is what has made my remarks so confusing to Dr Farber and doubtless to myself as well

radiologists in this audience so I shall have to constitute myself the international defender of radiology for the evening and say that I think most radiologists disapprove of the indiscriminate and drastic and excessively early use of radiotherapy in the management of such cases

All too often however the real situation is that the patient is referred by a physician whether it be the hematologist or any other physician not for the opinion of the radiotherapist as to whether radiation therapy is needed or not, but with what amounts virtually to a prescription an order if you will for radiotherapy leaving the radiotherapist in the difficult position either of treating the patient or making a fool out of the referring physician

Second I think it would be very misleading to this audience if the place of radiotherapy were not clearly stated in relation to the various agents that have been discussed this evening as things stand at the present time I realize that radiotherapy has been with us for many years and the novelty has worn off and certainly I do not begrudge any of you gentlemen the intellectual stimulation and pleasure that comes from working with these novel agents and evaluating them I think it is only fair to the audience however to summarize the situation by saying that at the present moment the single most consistently useful agent in the management of the chronic leukemias and lymphosarcomas and Hodgkin s disease is still radiation therapy in one form or another

Finally we have had occasion in a small handful of cases to bring patients with chronic myelocytic leukemia under remission with radiotherapy and then to follow this up with maintenance doses of either urethan or Myleran Our experience is so limited that I don t believe we can draw any conclusions except that the remissions have seemed to be of unusually long duration I wonder if any of the panelists have had any experience with this combination, and I also want to ask Dr Dameshek whether his statement that Myleran is more convenient to use than radiation therapy in the management of chronic myelocytic leukemia relates to the fact that he does not have an x ray machine in his office or that he sincerely believes that Myleran is the better agent

Chairman Bethell By the title of this discussion, which has to do with "new chemical agents" the topic of radiation therapy is largely excluded Nevertheless I tried without much success to induce our panelists to express opinions on the relative merits and the appropriate situations for the use of drugs and ionizing radiation

I don t think you meant to imply did you Dr Kaplan that the

alopecia and two peculiar instances of salivary gland aplasia with complete dryness of the mouth yet in one instance there was intense lacrimation The patient a woman had this condition for over a year—she had received in the course of 18 months of a rather active leukemia *1600 mg of Myleran—and at necropsy there was no evidence of any* aplasia of the marrow We have seen two instances of an unusual type of cardiac failure of course we don t know whether or not this is related to the Myleran

I should like to close with a suggestion I would not minimize the *value of $P^{3}$ and x ray therapy in combination with drugs particularly* in the treatment of chronic leukemias I know this is intended to be a panel on chemotherapy but I think the judicious use of $P^{32}$ and x ray therapy sometimes has a very definite place in the therapy of leukemia

Chairman Bethell Dr Storti your name has been associated with the use of some of these compounds particularly Colcemid Would you like to comment on your experience with Colcemid?

*(Dr Storti replied in French )*

Chairman Bethell I shall ask Dr Moeschlin to summarize in Eng *lish Dr Storti s remarks*

Dr Moeschlin I am sorry you didn t all understand but Dr Storti *stated that in his experience Colcemid is very useful in some cases of* Hodgkin s disease when all the other drugs have proved to be less effective He points out however that one has to be very careful in controlling granulocytes because in these cases granulocytopenia may occur and it may be induced by very small doses especially since in many of these advanced cases there is already leukopenia

Chairman Bethell Are there any other questions or comments?

Dr H S Kaplan (San Francisco California) Since Dr Bethell raised the question of the history of colchicine therapy I might first *state parenthetically* that the initial work on the treatment of experimental leukemias with colchicine was done 19 years ago by the modest gentleman on my right Dr Arthur Kirschbaum

What I really wish to do *is to bring up two agents one of which has* not been mentioned except disparagingly until Dr Bierman spoke namely irradiation and the second urethan which has not been mentioned at all this evening

I deeply regret Dr Moeschlin s comment about the use of radiotherapy in *chronic lymphatic* leukemia I don t know if there are other

Dr. George J Fuhrman (New York City) Despite the relative non specificity of the folic acid antagonists and other antimetabolites they nevertheless have a great deal of effectiveness in many of the acute leukemias This effectiveness must be due in some part at least, to the great avidity of these cells for the metabolite and hence also for the antimetabolite Indeed I believe Dr Bethell and his group have shown that certain leukemic cells possess higher concentrations of folic acid than do normal cells of the same series

The first part of my question is this Is it possible to take an acute leukemia and push it still further—that is to say to increase the great avidity of these cells for metabolites and antimetabolites to an even greater degree than existed prior to interference?

Second if this is possible and if an antimetabolite is administered at the time when the increased avidity exists would it then not be possible to have a cell which is even further sensitized to the action of the antimetabolite and even more specifically so and thus perhaps either reduce the levels of antimetabolite needed in the patient or reduce the toxic side effects? There would thus be envisioned hypothetically a type of cancer chemotherapy combination treatment not like that suggested by Dr Burchenal which consists of two blocking agents but rather a carefully timed combination of a provocative agent to stimulate the intake of a metabolite followed at the proper moment by the antimetabolite

Chairman Bethell I think *the same reasoning may* be applied to the use of cytotoxic agents such as the enhanced introduction of radioactive materials into cells through special means as has been suggested by Dr William Adams in Los Angeles He proposes to induce a phosphorus diuresis with loss of phosphorus from malignant cells and subsequent increased uptake of phosphorus as carrier for $P^{3}$ I am afraid however that such considerations take us beyond the scope of the discussion this evening Are there other questions or comments?

Dr. William R Best (Chicago Illinois) One of the methods of treatment in acute leukemia is to give the drug until a severe leukopenic condition is reached and then to continue it hoping to increase the likelihood of remission Most of us I think are afraid to do that I wonder if we could have some comment from the panelists on how they feel about this point of view

position of the radiotherapist is analogous to that of the dispensing pharmacist? I am sure that all the members of this panel have access to radiation therapy and that we take advantage of that access and that we have amicable and mutually satisfying relationships with our radio therapists Moreover included in radiation there is of course the use of radioactive isotopes which many of us actually control So I do not believe we are really talking about rival therapies or therapists I certainly do believe that the judicious use of the agents which we have discussed tonight in conjunction with radiation constitutes the best forms of treatment that we have available for most cases of chronic leukemia and lymphoma Dr Dameshek would you like to comment on the subject since reference was made to your expressed views?

Dr Dameshek Of course I would agree with Dr Kaplan There is no question but that x ray therapy in cases of chronic lymphocytic leukemia and chronic granulocytic leukemia is a highly useful procedure I doubt that as yet we have any really good statistics as to the effective life of an individual with chronic lymphocytic leukemia or chronic granulocytic leukemia as treated by x ray alone or by x ray and urethan or by Myleran alone

It is possible that x ray alone might be just as effective as Myleran alone In our experience it is nice to have an agent—a pill—that a patient can take by mouth which will act as effectively as x ray therapy in cases of chronic granulocytic leukemia Our remissions are just as good The patient feels just as well The spleen regresses just as satis factorily And I like Myleran because of the ease of therapy Further more there is the matter of divided responsibility We don t like to ask the radiologist to function merely as a technician and furthermore we like to control our own patients so we give the patient a pill and it works remarkably well Whether it is better than x ray I can t say I have the personal impression that it is better but that question is undecided

Concerning urethan prior to the introduction of Myleran we treated all our patients who had chronic granulocytic leukemia with x ray first reducing the leukocyte counts to reasonably low levels and then in stituting urethan as maintenance therapy We have since discontinued that practice I think we should have mentioned urethan with par ticular reference to chronic plasmocytic leukemia which is another name for multiple myeloma Multiple myeloma, I feel is chronic plasmocytic leukemia—more or less aleukemic to be sure Urethan is relatively useful in that disorder perhaps more valuable in Durham than it is in Boston but still relatively useful

In the early report by Petrakis on the use of Myleran one of the patients died of bronchogenic carcinoma

I should like to ask if anyone here has had reason to suspect that the use of Myleran might possibly be associated with a second tumor?

CHAIRMAN BETHELL Dr Wintrobe will you reply?

DR WINTROBE With regard to Dr Schilling s question my answer would be that we have not had that experience and I wonder if this association is not a matter of coincidence I have seen patients with acute leukemia who have had other tumors and their occurrence was at such a time that one could not attribute it to chemotherapy Further more on theoretical grounds would one not expect that a longer time of exposure to the chemotherapeutic agent would be necessary to produce the tumor if the drug were the cause? If we consider some of Haddow s experiments with these agents in producing tumors I wonder if that possibility doesn t seem more likely and that the answer to your question is that such a relationship is coincidence rather than cause and effect Of course I don t know the final answer

DR MOISES CHEDIAK (Havana Cuba) I should like to ask the panel about Actinomycin C and their experience with the use of this drug in some of these diseases and also about colchicine in the treatment of Hodgkin s disease

I should like to ask Dr Dameshek in view of his recent visit to Japan what is the present status of Neomycin and Sarcomycin in the treatment of these malignancies?

Finally I should like to hear a word about Fowlers solution which was not mentioned Is there still a place for this agent in maintenance of remissions or must it be discarded from our armamentarium?

CHAIRMAN BETHELL Arsenic in the form of potassium arsenite or Fowler s solution was the first chemotherapeutic agent introduced in 1865 used in the treatment of chronic leukemia with success and of course it is still effective We believe however that the compounds we have been talking about have advantages over Fowler s solution in terms of ease of control length of remission and avoidance of the undesirable side effects of prolonged administration of arsenic

I should like Dr Dameshek to answer the question addressed to him by Dr Chediak

DR DAMESHEK Actinomycin C is an antibiotic developed by Professor Waksman and then was taken up fairly recently in Germany

CHAIRMAN BETHELL I don t know to whom to address that question Would you like to address the question to someone in particular?

DR BEST Perhaps Dr Farber would be the best one to answer it

DR FARBER The rule we follow is this As long as the leukopenia is explained on the basis of replacement of the bone marrow by leukemic cells we continue to treat That rule has been a safe one to follow

CHAIRMAN BETHELL Dr Burchenal do you want to comment on this point?

DR BURCHENAL I might say that Dr Byron Hall has used mercapto purine in much larger doses than we have and his principle was to pay no attention to the white count but to treat until the marrow became somewhat aplastic As soon as the marrow became seriously aplastic he stopped therapy He got better results in adults with these higher doses of mercaptopurine than we could ever get with our more con servative dosage

In answer to Dr Fuhrman the only situation I know of where there has been some attempt at prior conditioning of the leukemia has been in connection with work done by Dr Lloyd Law I will take a chance that I am saying the right thing He is in the audience and can correct me if I am wrong Dr Law treated mouse leukemia with azaguanine until it became resistant to or dependent on that drug and then he ob served a markedly increased sensitivity to amethopterin I think he developed a similar situation by conditioning with mercaptopurine The mercaptopurine resistant cell was very sensitive to amethopterin We think maybe we have seen this phenomenon to a certain extent in some of the patients particularly in the adult group who were treated first with mercaptopurine and who then seemed to respond better to amethopterin than we would ordinarily have expected Dr Ellison has been concerned with this phase of the work

DR ROBERT F SCHILLING (Madison Wisconsin) It is common knowl edge that agents used in the treatment of leukemia as well as cancer chemotherapy in general including observations on experimental ani mals frequently have the potentiality of inducing or producing tumors In the patients we have treated with Myleran two out of twenty three have developed second seemingly unrelated malignancies One had a bronchogenic carcinoma and the other had a glioblastoma multiforme These neoplasms were the causes of death in the two patients

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for the therapy of Hodgkin s disease We did some studies with this material in Hodgkin s disease but observed absolutely no effect in a series of five cases So as far as I am concerned it has no value I know Dr Farber also has had some experience with it

As far as Neomycin and the other drugs used in Japan are concerned I am sorry but I didn t get to learn what the Japanese are doing in this connection

DR FARBER We have not studied Actinomycin C in man That has been studied extensively by French and German workers and they have reported effects worthy of comment at least in Hodgkin s disease and chronic lymphocytic leukemia and lymphosarcoma as well as in scat tered forms of carcinoma There is no effect in acute leukemia

We have studied rather extensively in the past two years Actinomycin D a different material from Actinomycin C and also an antibiotic originated by Professor Waksman This compound has no effect at all in acute leukemia We are studying its effect in chronic lymphocytic leukemia Hodgkin s disease and lymphosarcoma and in certain of the epithelial tumors but our observations are much too recent to be commented on any further

DR BURCHENAL As far as Sarcomycin is concerned Dr Gordon McGill of our group treated a few cases and Dr Gellhorn had a series of nine more some twenty five in all We gave doses up to as much as 8 g a day intravenously which is much more than the Japanese usually employed Their dosage I think was rarely above 2 g a day We saw no significant objective effects That is correct for your series too is it not Dr Gelhorn?

DR GELLHORN Yes

CHAIRMAN BETHELL Before adjourning the discussion I should like to thank the members of the panel and the participants from the floor for their enlightened and stimulating contributions and I wish to re emphasize that in my view we are justified in continuing our work on chemical agents in the treatment of the leukemias in a spirit of optimism and with the sound expectation that we have a great deal to look forward to in the future

The meeting is adjourned

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